

Influence of cyclic changes in ovarian hormones on resting metabolic rate and appetite in women

Marta Campolier Bassaganyas (2016)

<https://radar.brookes.ac.uk/radar/items/f4950b28-edd4-4dd7-8164-a839aca80d6b/1/>

Note if anything has been removed from thesis:

Copyright © and Moral Rights for this thesis are retained by the author and/or other copyright owners. A copy can be downloaded for personal non-commercial research or study, without prior permission or charge. This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the copyright holder(s). The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the copyright holders.

When referring to this work, the full bibliographic details must be given as follows:

Campolier Bassaganyas, M (2016)

Influence of cyclic changes in ovarian hormones on resting metabolic rate and appetite in women

PhD, Oxford Brookes University

**Influence of cyclic changes in ovarian hormones on
resting metabolic rate and appetite in women**

Marta Campolier Bassaganyas, MCB

PhD

2016

Influence of cyclic changes in ovarian hormones on resting metabolic rate and appetite in women

Marta Campolier Bassaganyas

Oxford Brookes University

A thesis is submitted in partial fulfilment of the requirements of the award of
Doctor of Philosophy

January 2016

Contributions / Authors work

This thesis is result of the author (Marta Campolier)'s work in terms of design, data collection and edition. The only exemption is the analyses of the breath samples on the isotope mass spectrometer which were sent to be tested in an external laboratory at the National University of Ireland (chapter 5 -study 3-). Nevertheless, the author interpreted and worked from the raw output to obtain the different gastric emptying parameters.

Acknowledgments

After three years and a half of moments of stress, excitement, disappointment, adventure, but most of all, hard work, one can only realise that this journey would have not been possible without the help, patience and guidance of many others.

To start with, I would like to thank Oxford Brookes University and the Nigel Groome studentship fund for giving me the opportunity to extent my career in research. Secondly, I am grateful to my three supervisors, Dr Lightowler, Thondre and Clegg for their work and time invested on me. From the interview for the position to the day of the submission, one and each of them has contributed to the essential guidance and the elaboration of my journey. Furthermore, other lectures of the faculty like Dr Ryan Pink and Wakefield Morys-Carter must be thanked for their assistance in the understanding of the ELISA and the statistical analyses.

I could not forget to appreciate Julieth Paola Suilaiman Rojas, Cosette Floutier, Elina Maudet and Ali McIntosh, for their assistance in the collection of data, as well as to all my participants of the different studies, without whom this research would have not been possible.

In addition, I could not be more grateful to my friends and colleagues in the department:

- Sarah Hillier, for the mentoring on the venepuncture technique and many other tips related to the menstrual cycle–related research.
- Shelly Coe, Sarah Kennedy and Alaeddine el-Chab, for the hours of PhD work that we have shared where complaints, laughter and hugs have always been welcomed.
- To the most amazing research assistants and lab technicians (as well as great friends) one would ever wish to have: Patricia Shaw, George Saltaouras, Sarah Warner and Sarah Graham, thank you for always being there and for listening and helping in everything you could.

Without doubt, I must thank my family who despite being far away from Oxford, they have always been supportive and loving whenever I needed it.

Finally, a special thank you to my husband Barrie. I greatly appreciate all the help, patience, care and love that you have given throughout this journey, without you this wouldn't have been the same.

Conference abstracts

Campolier M, Thondre S and Lightowler H (2015) Resting metabolic rate and the menstrual cycle. *Proceedings of the Nutrition Society*. Cambridge University Press 74(OCE1): E29.

Campolier M, Thondre S, Clegg M and Lightowler H (2015) Validity of predictive equations for estimating resting metabolic rate in women during the different phases of the menstrual cycle. *Proceedings of the Nutrition Society*. Cambridge University Press 74(OCE4): E268.

Abstract

Energy balance, when energy intake (EI) equals energy expenditure (EE), is key in the maintenance of body weight and composition. Pre-menopausal women seem to experience fluctuations on both sides of the energy balance equation and this seems to respond to changes in the ovarian hormones, estradiol (E_2) and progesterone (P_4) within a menstrual cycle (MC). In light of the current prevalence of overweight and obese in the female population, it seems imperative to have a better understanding of the influence of the ovarian hormones on energy regulation in pre-menopausal women.

This thesis aimed to: examine the fluctuations in salivary and plasma ovarian hormones within a MC in naturally cycling (NC) women and hormonal contraceptive (HC) users; investigate whether resting metabolic rate (RMR) significantly changed throughout the MC in NC women and in HC users; explore the association between RMR and the ovarian hormones; and investigate whether appetite responses to the same breakfast changed throughout the MC phases.

Ovarian hormones in plasma experienced a greater fluctuation than in saliva within a MC and this affected the correlation and the ratio between the two collection specimens. Increases in RMR in the luteal (LPh) compared to the other MC phases in NC women were observed, but were not statistically significant despite showing clinically meaningful fluctuations. Salivary P_4 contributed to the variance of RMR seen in the LPh. Finally, gastric emptying (GE) time and PYY response to a standardized breakfast changed significantly across the MC; the LPh had the fastest GE time and the smallest PYY response of all the phases. P_4 and E_2 - P_4 ratio were significantly correlated to GE time.

The findings suggest that NC women might experience changes in their inherent regulatory mechanisms by which energy homeostasis is achieved within a MC. Whether these regulatory changes are beneficial or not in the maintenance of body weight in the long term remains unknown.

Table of contents

Contributions / Authors work	I
Acknowledgments	II
Conference abstracts	III
Abstract	IV
Table of contents	V
List of tables	IX
List of figures	XI
Abbreviations	XIII
1. Literature review.....	1
1.1. The menstrual cycle: overview.....	1
1.1.1. The ovarian hormones: estradiol and progesterone	1
1.1.2. Hormonal contraceptives (HC).....	3
1.1.3. Methods for monitoring the MC phases	4
1.2. Energy balance: overview.....	9
1.2.1. Energy expenditure: resting metabolic rate.....	9
1.2.2. Energy intake and appetite regulation	17
1.3. Summary and aims	24
2. Methods	25
2.1. Ethical approval	25
2.2. Participant recruitment and screening.....	25
2.3. Baseline characteristics	26
2.3.1. Lifestyle questionnaire	26
2.3.2. Body composition assessment	27
2.4. Blood sample collection and analyses	27
2.4.1. Blood collection and storage.....	27
2.4.2. Plasma E_2 and P_4 measurement (Study 1, 2 and 4).....	28
2.4.3. PYY (total) measurement (Study 3).....	29
2.5. Saliva sample collection and analyses (Study 1).....	32
2.5.1. Saliva collection and storage	32
2.5.2. Salivary E_2 and P_4 measurement.....	32

2.6.	RMR and substrate metabolism assessment (Study 2).....	37
2.6.1.	<i>RMR measurement</i>	37
2.6.2.	<i>RMR measured vs. RMR predicted</i>	38
2.6.3.	<i>Substrates metabolism measurement</i>	38
2.7.	Gastric emptying assessment (Study 3)	40
2.7.1.	<i>Breakfast characteristics</i>	40
2.7.2.	<i>Breath samples collection</i>	41
2.7.3.	<i>Gastric emptying measurement</i>	41
2.8.	Food intake assessment (Study 3)	44
2.8.1.	<i>Food diaries</i>	44
2.8.2.	<i>Ad libitum food intake</i>	44
2.9.	Appetite sensations assessment (Study 3)	47
2.10.	Physical activity assessment (Study 3).....	47
2.11.	MC phases definition	48
2.12.	Statistical analyses.....	49
3.	Ovarian hormones in plasma and saliva during the MC (Study 1).....	50
3.1.	Background	50
3.2.	Methods.....	51
3.2.1.	<i>Protocol</i>	51
3.2.2.	<i>Calculations and statistical analyses</i>	51
3.3.	Results.....	52
3.3.1.	<i>Participants characteristics</i>	52
3.3.2.	<i>Changes in ovarian hormones across the phases of the MC</i>	53
3.3.3.	<i>Ratios and correlations between specimens</i>	56
3.3.4.	<i>Ovarian hormones analysed as averages of all the data points</i>	58
3.4.	Discussion	59
4.	RMR and substrate metabolism during the MC (Study 2).....	63
4.1.	Background	63
4.2.	Methods.....	64
4.2.1.	<i>Protocol</i>	64
4.2.2.	<i>Calculations and statistical analyses</i>	65
4.3.	Results.....	66
4.3.1.	<i>Participants characteristics</i>	66
4.3.2.	<i>RMR</i>	67

4.3.3.	<i>Ovarian hormones</i>	70
4.3.4.	<i>RMR and ovarian hormones in the LPh</i>	70
4.3.5.	<i>Substrate metabolism</i>	72
4.3.6.	<i>RMRp vs. RMRm</i>	73
4.4.	<i>Discussion</i>	76
4.4.1.	<i>RMR throughout the MC</i>	76
4.4.2.	<i>RMR variability</i>	77
4.4.3.	<i>Determinants of RMR</i>	78
4.4.4.	<i>Substrate metabolism</i>	80
4.4.5.	<i>NC vs. HC users</i>	80
4.4.6.	<i>RMRp vs. RMRm</i>	82
4.4.7.	<i>Limitations</i>	83
4.5.	<i>Conclusion</i>	84
5.	<i>Appetite responses during the MC (Study 3)</i>	85
5.1.	<i>Background</i>	85
5.2.	<i>Methods</i>	86
5.2.1.	<i>Protocol</i>	86
5.2.2.	<i>Calculations and statistical analyses</i>	88
5.3.	<i>Results</i>	89
5.3.1.	<i>Participants characteristics</i>	89
5.3.2.	<i>MC characteristics and ovarian hormones</i>	90
5.3.3.	<i>GE</i>	91
5.3.4.	<i>Total PYY</i>	91
5.3.5.	<i>Satiety ratings</i>	94
5.3.6.	<i>Ad-libitum, post-lunch and averaged food intake</i>	94
5.3.7.	<i>Relationships between PYY, GE, appetite feelings and EI</i>	96
5.3.8.	<i>Relationships between appetite markers and the ovarian hormones</i>	96
5.4.	<i>Discussion</i>	96
5.4.1.	<i>GE throughout the MC</i>	96
5.4.2.	<i>PYY throughout the MC</i>	97
5.4.3.	<i>Ovarian hormones associations</i>	98
5.4.4.	<i>Food intake and appetite sensations throughout the MC</i>	99
5.4.5.	<i>Limitations</i>	100
5.5.	<i>Conclusion</i>	101

6. General discussion and future research102

6.1. Limitations, implications, and future research105

References107

Appendices120

List of tables

Table 2.1. Validated predictive equations for female adults (EFSA 2013)	39
Table 2.2. Food composition of the breakfast (with milk)	41
Table 2.3. Equations used to calculate the different GE parameters (Ghoos et al 1993, Schommartz et al 1997)	43
Table 2.4. Foods available in buffet lunch with nutritional composition per portion provided	46
Table 3.1. Formulation of the different hormonal contraceptives used by participants.	52
Table 3.2. Baseline characteristics of the NC and HC groups.	53
Table 3.3. Ovarian hormone levels in plasma and saliva as an average of the MC in the NC and the HC group.	53
Table 3.4. Correlations between saliva and plasma for E ₂ and P ₄ levels in the different phases of the MC in the NC group.	57
Table 3.5. Correlations between saliva and plasma for E ₂ and P ₄ levels in the HCs group and in their different stages of the MC in the HC group.	57
Table 3.6. Ovarian hormone levels in plasma and saliva as an average of all data points NC and the HC group of the MC.	
Table 3.7. Saliva:plasma ratio for E ₂ and P ₄ in the different phases of the MC in NC from all data points.	
Table 4.1. Formulation of the different hormonal contraceptives used by participants.	66
Table 4.2. Baseline characteristics of the NC and HC groups.	67
Table 4.3. RMR at the different phases of the MC in the NC group.	68
Table 4.4. RMR at the different stages of the MC in the HC group.	68
Table 4.5. Ovarian hormone levels in plasma and saliva at the different phases of the MC in the NC group	70
Table 4.6. Correlations between RMR (kcal/d and kcal/kg/d) and hormone levels and body composition in the LPh	71
Table 4.7. Correlations between P ₄ levels and body composition in the LPh.	72

Table 4.8. Substrate oxidation rates (g/min) and contribution (%) in the MC of the NC and HC groups	72
Table 4.9. Substrate oxidation rates in the different phases of MC in the NC and HC groups	71
Table 4.10. Mean bias (\pm SD) and accurate predictions (%) of the predictive equations from RMRm in the MC.	73
Table 4.11. Bias (%) of the predictive equations from RMRm as an average of the phases of the MC in the NC group (means \pm SD).	74
Table 5.1. Participants' characteristics at baseline.	90
Table 5.2. GE parameters shown in minutes for MPh, FPh and LPh.	91
Table 5.3. Food intake during and after the <i>ad libitum</i> lunch and as an average of the three measured days in each MC phase	95

List of figures

Fig 1.1. Steroid hormone biosynthesis from (Hu et al 2010).	2
Fig 2.1. Collection of a blood sample from a cannula: (A) Flushing sodium chloride into the system with to ensure patency of the cannula (B) Extraction of a sample with the syringe to ensure the collection of a blood sample clean of sodium chloride (C) Extraction of the blood sample with a tube (D) Flushing into the system to maintain the cannula patent until the collection of the subsequent sample.	28
Fig 2.2. Plate layout for PYY assay	29
Fig 2.3. Serial dilution for PYY standards	30
Fig 2.4. Example PYY 4parameter curve fit	31
Fig 2.5. Plate layout for saliva E ₂ an P ₄ assays	32
Fig 2.6. Serial dilution for E ₂ standards	33
Fig 2.7. Example estradiol 4-parameter curve fit	34
Fig 2.8. Serial dilution for P ₄ standards	35
Fig 2.9. Example progesterone 4-parameter curve fit	36
Fig 10. Measurement of RMR. (A) First 30 min lying down for stabilisation (B) Last 30 min under the canopy to measure RMR	37
Fig. 2.11. Breath sample collection	41
Fig 2.12. Octanoic acid structure (PubChem (n.d.))	41
Fig 2.13. GE time points (Clegg and Shafat 2010)	42
Fig 2.14. Buffet lunch (non-vegetarian)	45
Fig 2.15. Example Visual Analogue Scale	47
Fig. 3.1. E ₂ (A) and P ₄ (B) levels in plasma and saliva in the different phases of the MC in the NC group (means ± SE)	54
Fig 3.2. Examples of plasma (A, B) and salivary (C, D) ovarian hormones throughout the MC in a NC participant (right) and a HC participant (left). <i>Dashed line indicates ovulation in NC woman; highlighted days correspond to the ON stage in HC user</i>	55

Fig. 3.3. E ₂ (A) and P ₄ (B) levels in plasma and saliva in the different phases of the MC in the HC group (means ± SE)	55
Fig 3.4. Saliva:plasma ratio for E ₂ and P ₄ in the different phases of the MC in the NC (A) and the HC (B) groups (means ± SE)	56
Fig 3.5. Correlations between plasma and salivary E ₂ (A) and P ₄ (B) concentrations in the LPh	57
Fig 4.1. Averaged RMR (kcal/d) per individual and phase in the high CV and low CV groups of NC women	69
Fig 4.2. RMR difference (Δ) between phases of the MC (kcal/d) calculated as (A) <i>FPh</i> – <i>MPh</i> , (B) <i>LPh</i> – <i>FPh</i> and (C) <i>LPh</i> – <i>MPh</i> . (■) are medians of the differences in RMR between phases.	69
Fig 4.3. Substrate contribution (%) to energy expenditure during the phases of the MC in the NC group (A) and in the ON and OFF stages of the HC group (B).	73
Fig 4.4. Accuracy of RMRp in the different phases of the MC for the ten predictive equations in the NC group. <i>HB</i> , Harris Benedict; <i>Schod</i> , Schodfield.	74
Fig 4.5. Difference between RMRm and RMRp predicted by the Henry_1 equation against RMRm in the MPh (A), FPh (B) and LPh (C).	75
Fig 5.1. Example experiment time line during a MC.	87
Fig 5.2. Timeline of events during each of the testing days in the laboratory.	88
Fig 5.3. E ₂ and P ₄ concentrations in the different phases of the MC (means ± SD).	90
Fig 5.4. Example of a participant's breath ¹³ CO ₂ curves, expressed in excess atom fraction over the time following each of the three phases of the MC.	91
Fig 5.5. PYY levels at each time point in the different phases of the MC (Means ± SD).	92
Fig 5.6. Change in PYY levels from baseline to each time point in the different phases of the MC (Means ± SD).	92
Fig 5.7. Total PYY AUCs at t = 60, 120, 180 and 240 in the different phases of the MC (Means ± SD). ^a Significantly different to the LPh within the same time AUC.	93
Fig 5.8. Total AUCs (mm/min) at t = 60, 120, 180 and 240 in the different phases of the MC for the four appetite sensations questions: (1) 'How hungry do you feel?' (A), (2) 'How full do you feel?' (B), (3) 'How strong is your desire to eat?' (C) and (4) 'How much food do you think you can eat?' (D). (Means ± SD)	95

Abbreviations

• AgRP	Agouti-related peptide
• ARC	Arcuate nucleus
• AUC	Area Under the Curve
• BBT	Body basal temperature
• BMI	Body mass index
• BW	Body weight
• C	Carbons
• CART	Cocaine-amphetamine regulated transcript
• CBG	Cortisol binding globulin
• CCK	Cholecystokinin
• CHO	Carbohydrates
• CIA	Chemiluminescence immunoassay
• CNS	Central nervous system
• CV	Coefficient of variation
• E ₂	Estradiol
• EE	Energy expenditure
• EI	Energy intake
• EIA	Enzyme immunoassay
• ELISA	Enzyme-linked immunoassay
• FFM	Fat free mass
• FM	Fat mass
• FPh	Follicular phase
• FSH	Follicle-stimulating hormone
• GLP-1	Glucagon-like peptide 1
• GnRH	Gonadotropin releasing hormone
• HC	Hormonal contraceptives
• HMRO	High metabolic rate organs
• HPG	Hypothalamic-pituitary-ovarian/gonadal
• LH	Luteinising hormone
• LHA	Lateral hypothalamic area
• LPh	Luteal phase
• MC	Menstrual cycle

- MCT Mid-chain triglyceride
- MPh Menstrual phase
- NC Naturally cycling
- NPY Neuropeptide Y
- OC Oral contraceptives
- OD Optical density
- P₄ Progesterone
- PA Physical activity
- PAI Physical activity index
- PAQ Physical activity questionnaire
- Pd-3- α -G Pregnanediol-3- α -glucuronide
- PMS Premenstrual Syndrome
- POMC Pro-opiomelanocortin
- PSST Premenstrual Syndrome Screening Tool
- PVN Paraventricular nucleus
- PYY Peptide YY
- RER Respiratory exchange ratio
- RIA Radioimmunoassay
- RMR Resting metabolic rate
- RMR_{adj} Adjusted RMR
- RMR_m Measured RMR
- RMR_p Predicted RMR
- SCN Suprachiasmatic nucleus
- SHBG Sex hormone binding globulin
- SMR Sleeping metabolic rate
- TEE Total energy expenditure
- VAS Visual Analogue scale
- VHN Ventromedial hypothalamic nucleus
- VO_{2max} Maximal oxygen uptake
- α -MSH α - melanocyte-stimulating hormone

1. Literature review

1.1. The menstrual cycle: overview

From the age of 12-13 years when the menarche, i.e. the first menstrual period occurs, until the age of 45-55 years when menopause begins, healthy women experience an ordered sequence of hormonal and morphological events that repeat every month with the aim to promote human reproduction; this is the menstrual cycle (MC).

The coordination of these events relies on a hypothalamic-pituitary-ovarian/gonadal (HPG) close relationship perfectly synchronised. As menstruation ceases at the beginning of a MC, the hypothalamus releases the gonadotropin releasing hormone (GnRH) to stimulate the anterior-pituitary to synthesise the luteinising hormone (LH) and the follicle-stimulant hormone (FSH). These two gonadotropins will induce the progressive production of oestrogen in the ovary which is the hormone responsible for the growth and maturation of the follicle containing the oocyte, hence why this is called the follicular phase (FPh). It can also be called the proliferative phase as it represents the time of repair and growth of the endometrium of the uterus post-menstruation. As estradiol concentrations reach the first peak during the MC they will induce a sudden and sharp rise in LH that will stimulate the release of the oocyte from the ovary to the fallopian tube, i.e. ovulation, within 24-48h. From what is left of the follicle in the ovary, i.e. the corpus luteum, the other ovarian hormone i.e. progesterone, along with oestrogen, is synthesised. Progesterone will be in charge of finalising the preparation of the endometrium by increasing the surface area of the endometrial glands and by stimulating the secretion of different nutrients that will allow the implantation and survival of the fertilised egg in the uterus; thus the post-ovulatory phase is called the luteal phase (LPh) or secretory phase. If the oocyte does not get fertilised, the cycle will finish by the cessation of the production of the ovarian hormones and the shedding of the endometrium (menses) which will subsequently stimulate the secretion of GnRH to begin with a new cycle (Ferin et al 1993).

1.1.1. The ovarian hormones: estradiol and progesterone

Each MC will last for approximately 28 days (usually between 22 and 36 days) and, according to the changes that occur in the ovarian hormones, it can be divided in three MC phases (1) the menstrual phase (MPh) when oestrogen and progesterone are at very low concentrations,

(2) the FPh when oestrogen levels gradually increase until a peak after which ovulation occurs and (3) the LPh in which oestrogen and progesterone progressively increase until they peak and then drop, provided the egg has not been fertilised.

Oestrogen and progesterone, (along with the other steroid hormones, such as androgens, glucocorticoids and mineralocorticoids), are the product of the different enzymatic changes originated from the 27 carbons (C) steroid hormone cholesterol. As shown in Fig 1.1, progestagens are derived from the first step in the conversion of cholesterol which results in 21C hormones from which the most predominant is progesterone (P_4). To obtain androgens, the conversion from progestagens by losing two carbons is required, thus androgens are made of 19C. Of all the androgens, testosterone (T) is the most potent but only androstenedione is found at similar, rather than lower, concentrations in women and men to serve as a precursor of oestrogens via direct aromatisation to oestrone (E_1) or through the conversion to testosterone first and then to estradiol (E_2) (Heffner and Schust 2014). Oestrogens are formed of 18C and E_2 is the most potent of all, hence its research interest. Although the gonad organs, i.e. the ovaries for women, are the main producers of the sex steroid hormones (progestagens, androgens and oestrogens) the adrenal gland also contributes in the production, mainly as a result of the synthesis of the corticoids (nevertheless, their function becomes more significant during pregnancy or at menopause).

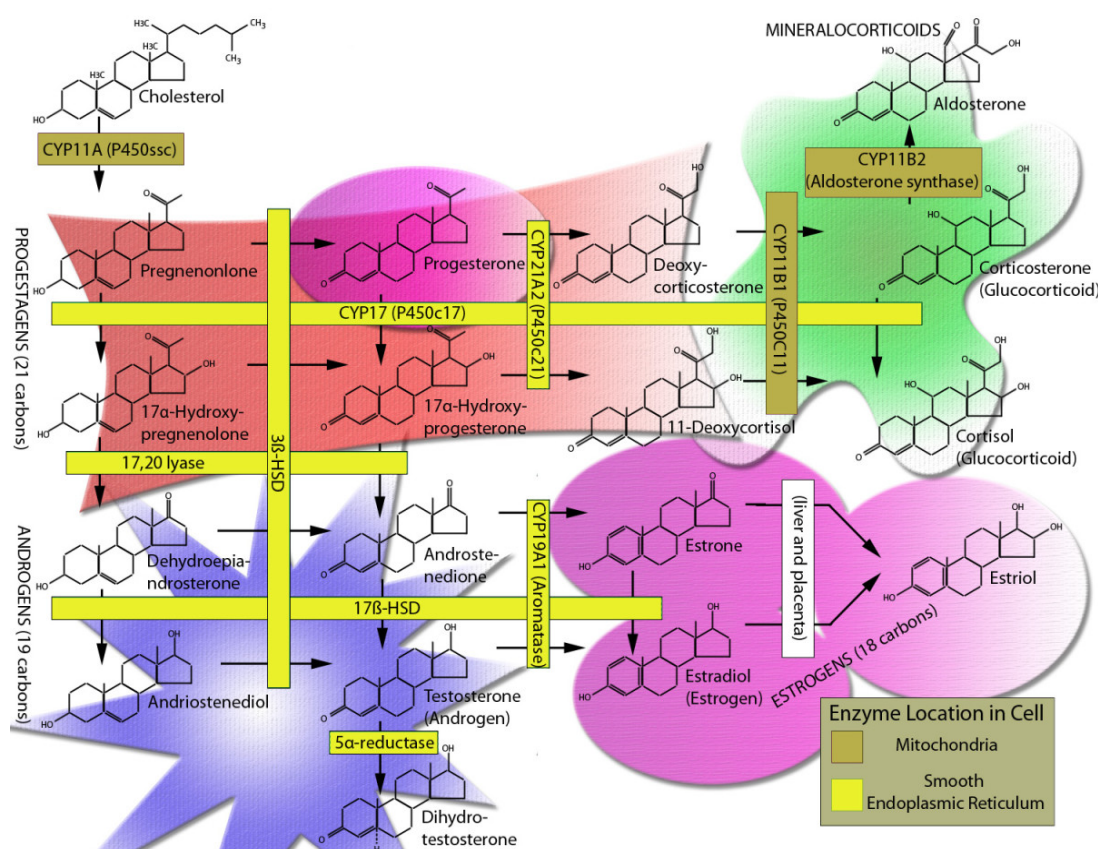


Fig 1.1. Steroid hormone biosynthesis from (Hu et al 2010).

The synthesis of oestrogens in the ovary is regulated by the response of the theca cells to the presence of LH, which will facilitate the conversion of cholesterol to androstenedione, as well as the aromatisation from androstenedione to oestrogen induced by the FSH in the granulosa cells.

The sex steroid hormones E_2 and T are transported in the bloodstream by the same carrier, namely the sex hormone-binding globulin (SHBG). In fact ~70% of the total of each of these hormones are bound to this globulin while 30% are carried in a weaker bond to albumin, thus allowing only 1-2% of the total concentrations free and ready to access to the target tissue (Tenovuo 1989). Similar relative concentrations of free P_4 are found in blood. Moreover, P_4 can also be weakly bound to albumin. However, about half of the conjugated- P_4 in blood is carried more strongly with another protein, namely the cortisol binding globulin (CBG), which is also used by cortisol (Bentley 1980).

1.1.2. Hormonal contraceptives (HC)

Since the late 1940's when Dr Carl Djerassi synthesized for the first time "the pill" that could "arrest ovulation" in women, many different approaches to women's birth control have been proposed and its acceptance and use within the society has changed enormously. In fact, the advances in technology have not only allowed the dose of the oral contraceptives (OC) to be less than a third of the original content, but also new hormone delivery systems e.g. contraceptive vaginal rings, patches, injections and implants, have been launched into the world market (Liao and Dollin 2012). Therefore, in 2006-07 76% of the women of 16-49y in Great Britain were using at least one form of contraception, of which 43% were some type of hormonal contraceptives (HC) (FPA 2007).

HC can be classified in two groups according to their composition. Firstly, the combined HC, which is the most commonly used and includes a portion of synthetic oestrogen (mainly ethinylestradiol) and another of a progestogen (which can be of many different forms). This category can be further categorised depending on the release pattern of the contained hormones throughout the MC: monophasic, biphasic and triphasic combinations. Monophasic combinations supply both hormones constantly at the same concentrations, whereas biphasic and triphasic combinations (less popular) provide two and three different dosages of the two hormones, respectively, throughout the MC. Finally, the progesterone-only HC is formulated

only by progestagens and it is less used, being mainly prescribed to women of a higher cardiovascular risk (Frye 2006).

HC action is primarily based on preventing the development of the follicle and thus ovulation by the induced inhibition of the FSH and LH secretion by the intake of exogenous hormones. Furthermore, they enhance their function by targeting peripheral parts of the ovaries such as the endometrium and the cervix by making the entry and implantation environment more hostile for the sperm (Monga and Dobbs 2011). As the two gonadotropins are inhibited, the synthesis of ovarian hormones is expected to be affected. The effect of HC on testosterone levels was examined in a systematic review and it was concluded that HC induced alterations in the free- and bound-testosterone levels, which were significantly reduced while their carrier i.e. SHGB, was significantly increased (Zimmerman et al 2014). Furthermore, significant increases in CBG have also been observed (Granger et al 2009), thus suggesting an impact on total and free ovarian concentrations.

1.1.3. Methods for monitoring the MC phases

Natural MC can vary widely from the typical “28-days” cycle described in text books in which ovulation occurs in day 14 and the FPh and LPh are equally distributed around this event. Indeed, the MC and its phases not only vary in length between individuals, but also within individuals. A study by Fehring et al (2006) demonstrated how ~43% of 141 women that were followed during ~5.2 MCs displayed an intra-cycle length variation of 7-14 days. Moreover, they showed that the variability of the length was mainly dependant on the length of the FPh rather than the LPh (34 vs. 9 % of the participants had a longer than seven day intra-phases variability for the FPh and LPh, respectively). These natural variations will have an impact on the design of any MC related study as these are indicating differences in the time exposure of each of the ovarian hormones per MC.

For the last decades, saliva samples have progressively become a common method used by researchers and clinicians to measure and monitor levels of hormones such as cortisol and sex steroids (Gröschl 2008). The benefits of this technique versus the traditional vein-puncture are the non-invasive, stress-free and easy to collect and store characteristics that define this method. Moreover, the salivary hormone levels solely provide the unbound (free of protein) portion of the total volume (Bellem et al 2011), these have shown a high correlation with the total or a portion (i.e. free or bound) of the hormone levels measured in blood (Hofman 2001).

For example, Bolaji (1994) showed that P_4 concentrations assessed in saliva and serum were highly correlated ($r = 0.974$, $p < 0.0001$) in a population of 23 healthy regular menstrual cycling women. Both samples were collected on a single day within 15 min at 7.00-10.00 am in a fasted state. Saliva was analysed using a non-isotopic solid-phase EIA (enzyme immunoassay). Although the results of this study showed the natural change of P_4 levels in saliva during the different phases of the MC (i.e. ≤ 250 and 251-885 pmol/l in the FPh and LPh, respectively), the author of the study failed to fully specify how the MC phases were assessed and the number of participants included in each phase. In addition, the findings might have been more persuasive if two measurements had been taken from the same participants, i.e. one in each of the phases of the MC.

In fact this is what De Boever et al (1986) did in their study in which seven women provided a saliva sample every two or three days throughout their MC. In this study, the researchers employed a direct solid-phase chemiluminescence immunoassay (CIA) to measure P_4 in saliva. The results confirmed that P_4 concentrations vary across the MC, i.e. 178 ± 46 , 313 ± 90 and 658 ± 166 pmol/l in the FPh (days -11 to -3), periovulatory period (days -2 to +1) and LPh (days +2 to +11), respectively, although they failed to state whether these differences were significant. Moreover, they also measured serum samples in two occasions during the FPh, and three times in the periovulatory and LPh. Similarly, they found a positive correlation between the 96 serum and saliva samples across the MC ($r = 0.88$ $p < 0.001$). Furthermore, this study showed that depending on the phase of the MC there was a different serum-to-saliva ratio, i.e. 5.1, 16 and 56 in the FPh, periovulation and LPh, respectively. However, the two different specimens were not taken simultaneously thus the values compared might have not taken into account possible diurnal differences and might have been biased by the averages obtained from every phase. Nevertheless, their findings concurred with Cedard et al's (1984) study in which 14 women simultaneously provided a pair of specimens, i.e. saliva and blood samples, every two days throughout their MC. In this case, the researchers employed a radioimmunoassay (RIA) for the analysis of the saliva samples. Their results showed that in the FPh P_4 levels in saliva were significantly lower than in the mid-LPh (48-254 and 184-566 pmol/l, respectively ranges). Furthermore, there was a positive correlation between salivary and plasma P_4 during the mid-LPh ($r = 0.63$) ($n = 39$) ($p < 0.001$). In agreement with De Boever et al (1986), this study showed that the saliva:plasma ratio changed substantially during the different phases of the MC i.e. 0.0532 and 0.0112 in the FPh and LPh.

Nevertheless, that is not in complete agreement with some other studies. In fact, a recent investigation by Konishi and colleagues (2012) demonstrated that this correlation is altered by an individual saliva-to-blood ratio. In this case, they compared cortisol and P_4 concentrations of saliva against serum obtained from finger pricks once a week for 4 consecutive weeks ($n = 48$) or daily for 40 consecutive days ($n = 10$) (a validation study of the finger prick blood samples had previously been conducted). The participants self-took both specimens simultaneously at the time of the day that suited them best (between 5.00am-6.00pm) and they stored the samples at home. Dried blood spot samples from the finger pricks were collected in a filter paper and analysed by EIA and saliva samples were collected from passive drool and analysed using EIA (by Salimetrics Salivary Assay kits). Both techniques showed a positive, but weaker correlation ($r = 0.345$, $p < 0.001$) compared to the serum-saliva relationships in the abovementioned studies. This may have been due to the type of assays employed in the different studies. Moreover, the correlation became stronger when studied in subgroups of high, medium and low- saliva-to-blood ratio ($r = 0.695$, 0.652 and 0.533 , respectively) ($p < 0.001$ for all). Finally, it is interesting to mention that saliva, but not plasma, P_4 levels were significantly higher early in the morning (5.00-9.00 am) than late in the morning (9.00am-12.00pm) or in the afternoon (12.00-6pm).

Similarly to P_4 , other research has been done on salivary oestrogen with the aim of monitoring the MC. For example, Worthman et al (1990) studied saliva E_2 levels intermittently during early to mid-FPh and then twice a day (at 9.00 and 21.00 h) at the late FPh until concentrations of E_2 peaked and ovulation was detected by ultrasound in 15 healthy women. Their results from RIA showed that E_2 peaked on day 14.4 (i.e. 1.2 days before ovulation) and this occurred on an individual time relative to ovulation. In addition, they also measured E_2 in saliva and plasma in a group of 18 women who were under ovulation-induction therapy in which both specimens showed a strong linear relationship with a saliva-to-serum ratio of 0.005 ± 0.003 . Nevertheless, they suggested that the fraction of free E_2 in plasma (assuming it was equivalent to salivary E_2) was not directly altered by the amount of total E_2 found in blood as there was no correlation between the saliva-to-serum ratio and total plasma E_2 .

Other findings by Lu et al (1999) showed that saliva E_2 peaked from 17 pmol/l (on day -10) to 45 pmol/l (on day -1) in seven women and that serum to saliva was positively correlated in all but one subject. However, results were solely statistically significant in five of the positive correlations. This agrees with the idea that remarkable individual differences can be encountered. However, it is worth bearing in mind that the serum samples were taken in a

fasted state in the morning whereas saliva samples were collected from unstimulated saliva at night to be finally analysed by double-antibody RIA with [125 I]E₂. This may have influenced the results as Bao and co-workers (2003) showed that free E₂, likewise P₄, suffers diurnal and ultradian rhythms. In this study 15 healthy Chinese women provided a saliva sample every 2h for four days (one day in each of the MC phases: MPh, late FPh, mid-LPh and late LPh) from 20:00-06:00 of the following morning. The collection method consisted of soaking a small polyester tampon with saliva and the analyses was performed by RIA. Their results showed that E₂ followed a rhythm with circadian and ultradian constituents that were fairly similar throughout the phases of the MC despite the differences observed between individuals. Nevertheless, the daily peak in oestrogen appeared to be later in the day in the MPh than in the late-FPh and, unexpectedly, no significant differences in the mesors ("average around which a variable oscillates") between phases were observed. This might be due to the fact that the saliva samples were taken solely one day in each of the MC phases and these were not correctly assigned, yet an ovary ultrasonic examination was performed and changes in the body temperature were monitored to ensure the phase of the MC, thus insignificant differences could not be explained.

Another important feature to take into account when measuring saliva E₂ and P₄ is that they may exhibit a similar effect to that observed in cortisol after waking up ("the cortisol awakening response"), i.e. awaking produces a neuroendocrine activation of the hypothalamus-pituitary-adrenal (HPA) and the suprachiasmatic nucleus (SN) that will cause an increase in cortisol levels after 30 and 60 min of waking up. Interestingly, and differently to what happens with cortisol, the rise in salivary P₄ and E₂ concentrations after awaking was only observed in natural regular cycling women whereas postmenopausal women showed no effect (Ahn et al 2011). Moreover, this response occurred simultaneously in the two hormones and with no differences throughout the MC phases. These findings may suggest that the diurnal patterns showed by Bao et al (2003) might have been confounded by alterations in the HPA and SN as participants were awoken every 2h during the sleeping time.

When reviewing the findings of the literature, it is crucial to determine whether salivary E₂ and P₄ concentrations are compared / correlated to the free portion in plasma (Cedard et al 1984, Evans 1986), or the total concentrations in serum (De Boever et al 1986, Bolaji 1994, Konishi et al 2012, Worthman et al 1990). Trying to correlate levels of unbound sex hormones (measured in saliva) against total concentrations (measured in plasma) might seem inappropriate, however, Evans (1986) found that the proportion of unbound P₄ in saliva and plasma was kept

constant throughout the phases of the MC (i.e. ~2 and ~90 %, respectively), and that there was a high positive correlation between the two. Conversely, he also showed that the ratio between these two changed depending on the phase of the MC i.e. 47.1 and 115 in the FPh and the LPh, respectively (Evans 1986), as previously shown with serum-to-saliva ratio (De Boever et al 1986). Therefore, he concluded that the alterations in this ratio between specimens might be due to a change in the ability of the steroids to cross the capillary membranes, rather than a change in the proportion of unbound hormones in blood.

In conjunction, these studies support the idea of using saliva sampling for E_2 and P_4 as a monitoring method of an individual's MC. However, more controversy exists when trying to extrapolate these values into plasma or serum concentrations. Not only might there be an individual saliva-to-blood ratio, but also this is affected by the phase of the MC. Moreover, the use of different techniques for the collection and analyses amongst these studies has contributed to the wide range of results regarding the concentrations of these hormones and their correlations in blood.

Nevertheless, saliva seems to be a reliable method of monitoring the ovarian function that also confers some advantages to other non-invasive techniques employed. For instance, when comparing daily saliva samples against early morning urine samples throughout the MC, peak P_4 concentrations in the LPh appeared in urine as pregnanediol-3- α -glucuronide (Pd-3- α -G) 1.8 days delayed compared to saliva peak P_4 (Adekunle et al 1989). This delay occurred as a consequence of the metabolic process that Pd-3- α -G needs to go through before appearing in urine, whereas blood hormones passively diffuse to the saliva matrix without having to be metabolised. However, concentrations of both specimens were significantly correlated.

Another non-invasive technique employed is the body basal temperature (BBT) which increases when P_4 is raised during the LPh. This was demonstrated by Lee (1988)'s study in which 13 naturally-cycling women and three using HC provided BBT (from rectal measurements) during 33h in each of the MC phases. The study revealed that BBT increased ~ 0.36°C in the LPh compared to the FPh in the naturally-cycling women, whereas no differences were observed in the HC users. Moreover, the change in BBT was simultaneous to a significant increase in the levels of salivary P_4 in the LPh compared to the FPh (526.4 ± 183.78 vs. 216.0 ± 70.57 pmol/l). Nevertheless, not all women experience an increase in BBT, even when ovulation has occurred (Moghissi 1976).

Therefore, in summary saliva collection seems to be not only a comfortable and non-invasive technique for the measurement of the ovarian hormones, but also it may offer a reliable method for distinguishing the different phases of the MC. Finally, to our knowledge, only a few studies have measured salivary P_4 and E_2 in women taking HC and none have examined the correlations between these and plasma concentrations.

1.2. Energy balance: overview

It is well known that metabolic diseases are a major public health concern due to their impact on society's life and economic cost (Yach et al 2006). Of the metabolic diseases, a main concern is the increasing epidemic of overweight and obesity present in the global population for the last decades. Recent estimated figures of the World Health Organization (WHO) showed that in 2014 1.9 billion adults aged ≥ 18 y were overweight, i.e. with a body mass index (BMI) of 25-30 kg/m^2 , and 11 and 15% of the male and female adults, respectively, were obese, i.e. BMI $\geq 30 \text{ kg/m}^2$ (WHO 2015). As the overweight population has increased, the amount of people dieting, i.e. to restrict food intake and control eating patterns, has been simultaneously raised. In 1998 it was estimated that 53% of the U.S overweight and obese women of ≥ 18 years attempted to lose body weight (BW) (Kruger et al 2004). Nevertheless, attempts to lose BW are not always achieved.

Energy balance, namely when energy expended equals energy intake, is the key to maintain metabolism in harmony and BW stable. In contrast, continuous imbalances between what is metabolised and what is ingested can induce changes in BW and body composition. It is suggested that women's metabolism can vary across the phases of the MC as their metabolomic profile of different metabolites (e.g. glutamine, lysine) in plasma is changed in the LPh compared to the MPh of the MC (Wallace et al 2010). Having a better understanding on how energy balance is achieved throughout the MC might aid the understanding on energy regulation in women.

1.2.1. Energy expenditure: resting metabolic rate

Energy expenditure (EE) is the sum of the resting metabolic rate (RMR), physical activity (PA) and the thermogenic effect of food (EFSA 2013). Of all these components, RMR (occasionally referred as basal metabolic rate -BMR-), i.e. the energy required by the body cells to maintain

postabsorptive homeostatic functions in resting subjects (Wang et al 2001), is the most important component of total EE (TEE) (i.e. ~70% TEE) (Payne and Waterlow 1971). Therefore, its determination is crucial in order to assess the daily individual energetic requirements, particularly in overweight and obese populations whose estimated RMR by prediction equations can be remarkably inaccurate in some cases (i.e. >10% difference to the actual RMR) (Frankenfield et al 2003). Consequently, measuring RMR using indirect calorimetry may enable us to have a better understanding of an individual's requirements. Differences in RMR are attributed to age, gender, race, PA and body composition (Arciero et al 1993, Speakman and Westerterp 2010).

Van Pelt et al (1997) studied RMR in pre- and postmenopausal women with the aim to demonstrate an age effect on RMR when adjusted by fat mass (FM) and fat free mass (FFM). Moreover, due to the potential influence of fitness on this parameter, the authors investigated this phenomenon by subdividing participants into sedentary and endurance trained groups. Aerobic fitness was assessed by measuring their maximal oxygen uptake (VO_{2max}). The MC was controlled by having the premenopausal women studied at the early FPh (days 1-10) and none of them was taking HC. Of the post-menopausal women, eight out of 15 in each subgroup was under oestrogen-based hormone supplements. Their results showed that adjusted RMR (RMR_{adj}) decreased by 10% in sedentary postmenopausal women compared to their younger counterparts. Conversely, there was no difference between pre- and postmenopausal endurance trained women. This suggests that PA status may modulate changes in RMR caused by aging. In fact, a significant correlation was found not only between RMR_{adj} and VO_{2max} ($r = 0.48$), but also VO_{2max} accounted for 35 % of the RMR_{adj} variance in women. This indicated that a higher VO_{2max} accomplished by long-term endurance training could potentially impair the age-decreasing effect on RMR_{adj} observed in sedentary women. Nevertheless, the authors failed to report whether there were any differences in RMR between the sedentary and endurance trained populations. Despite expecting significant differences between trained pre- and sedentary postmenopausal women, this might not have happened when comparing sedentary pre- against trained postmenopausal women. Therefore, although being physically active during youth and adulthood might be a good method to maintain a high RMR_{adj} , starting PA at a later stage, e.g. mid-thirties, might also have a positive impact on RMR_{adj} at later stages of life, thus it should be recommended to all women regardless their age.

Arciero et al (1993) demonstrated that women had 23% lower RMR than men in general, regardless of the age subgroup (i.e. pre- or postmenopausal women against younger or older

men). Moreover, when adjusting RMR with FFM, FM and aerobic fitness the differences were maintained, yet reduced due to their high contribution to RMR. Young men had 4% higher RMR_{adj} than premenopausal women and older men had 5% higher RMR_{adj} than postmenopausal women. The reason behind the gender difference despite controlling for body composition and fitness could not be explained by the study; however, the same group of researchers had previously demonstrated that Na^+-K^+ -ATPase activity, which is related to RMR, was reduced in women, thus potentially accounting for the difference. Finally, in agreement with Van Pelt et al (1997)'s results in the subset of endurance trained women, Arciero et al (1993) found no differences in RMR_{adj} between pre- and post-menopausal women. Nevertheless, this sample included women of a wide range of aerobic fitness levels, compared to endurance trained women in Van Pelt's study, although Arciero adjusted RMR with VO_{2peak} thus confirming the impact of age and physical fitness on RMR.

Forman et al (1998) investigated RMR in inactive obese African-American and Caucasian women and found that the former had a 9% lower RMR compared to the latter. The authors controlled age, body mass index (BMI), habitual PA, fitness status, and MC, thus it seemed conclusive that race could therefore make a difference in RMR, namely, obese sedentary African-American women appeared to be more energetically efficient than their Caucasian counterparts. Moreover, when RMR was controlled by FFM, the difference between the two increased to 12%, making evident the race-effect and this was attributed to metabolic differences between obese African-American and Caucasian women.

As aforementioned, understanding and calculating RMR in overweight and obese people is crucial to accurately address their enhanced energetic needs. Although one could assume that their higher BW would explain the greater RMR in adults, actually, FFM has been shown to be more strongly correlated to RMR than BW (Ravussin et al 1982). Therefore, on basis of a 2-compartment model of body composition with FM and FFM as parts of the total BW, different authors have tried to find an equation that better defines RMR. Nelson et al (1992) tested different mathematical models in a large population of adult men and women that were non-obese and obese (i.e. with a %FM > 20% in men and >30% in women) and demonstrated that FFM accounted for the majority of variance in RMR. Conversely, after controlling for FFM, differences in FM would have a minor effect on RMR. That was due to the higher relative metabolic rate of FFM over FM. However, yet the best predictive model contained both covariates because although FM might not have a great impact on RMR after controlling FFM in lean individuals, RMR differences between lean and obese stop being significant solely when

FM (36% difference between groups) was added into the equation. Nevertheless, that equation just accounted for the 74% of the RMR variance, thus, other possible parameters needed to be studied to fully predict RMR.

Supporting the impact of FM on RMR, Bosy-Westphal and colleagues (2008) demonstrated a concomitant increase of RMR in women with differing %FM. Nonetheless, it is worthwhile mentioning that differences in RMR between %FM categories within $>10\text{--}\leq 50$ %FM were no longer observed after controlling for age, FFM and FM. Furthermore, this study showed that FM impact in RMR increased with adiposity in agreement with Nelson et al (1992). However, in severely obese (> 40 %FM) and severely lean women (≤ 10 %FM), RMR did not relatively relay further on FM. Authors could not explain the reason behind the alterations on the relative impact of each body composition component in RMR.

Recently, Javed et al (2010) aimed to improve these predictive models by including the abovementioned variables (i.e. age, gender, race, BW and body composition) and adding FFM in its components, thus, the liver, kidney, spleen, heart and brain (i.e. high metabolic rate organs –HMRO–) in their specific relative metabolic rates into the predictive equation. Firstly, their results predicted ~68% of the RMR variance after including age, sex, race, BW and height. By changing BW and height to FM and FFM, the model improved to ~70% of the predicted RMR variance and sex became an insignificant variable. Moreover, by adding the HMRO of the trunk (thus, excluding the brain of HMRO) the model enhanced another 3% of the RMR variance and dismissed the statistical significance of race contribution to the predictive equation. Finally, the inclusion of the brain improved the predicted variance of RMR to ~75%. This suggested that sex, race and age impact on RMR actually responded to the differences in HMRO that occur with age and between sexes and races. Nevertheless, the last model still did not resolve the 25% remaining variance of RMR. Different possibilities could underlie this such as an individual RMR for each organ or the need to add other HMRO to the equation. Therefore, other studies are required to further understand the variables affecting RMR.

1.2.1.1. RMR during the MC

Although all the abovementioned factors that determine the RMR of a person are rather stable on a day-to-day basis, whether RMR is kept constant daily and more specific, throughout the course of a MC is the question that has brought discrepancies between studies for many years. Daily variability of RMR has been measured by looking at its coefficient of variance (CV) from

repeated measurements on the same subject. Despite the low values found in men i.e. 2.5-4.0 % (Henry et al 1989), wider ranges 1.7-12.0 % (Curtis et al 1996, Diffey et al 1997, Henry et al 2003) have been found in women which may be due to the potential effect that the MC may have on energy balance during phases of the MC.

In 1982, Solomon et al showed that BMR, collected for 10 min every other day during three MCs, was suppressed from menstruation to a week before ovulation but raised again in the LPh in five out of six women. The average CV between women was 12% with an individual CV ranging from 6-9 %, thus making evident the greater variance in women compared to men. Nevertheless, the authors did not provide the average values for each of the phases and the sample size was small ($n = 6$), thus making it challenging to assume that this occurs in the vast majority of women. Moreover, diet and PA were controlled and therefore whether these two components would have been altered in normal conditions throughout the MC and whether that would have affected their BMR patterns remain unknown.

In fact, this partly disagrees with Howe et al (1993)'s research as their first experiment revealed that 1h of indirect calorimetry showed no differences in RMR amongst the three MC phases in a healthy but within a wide range of BMI ($17.0\text{-}46.6 \text{ kg/m}^2$) population of 14 premenopausal women under free-style conditions. Moreover, this lack of change was still present when controlling for BW or BMI. However, in the second experiment with another group of 12 women the authors found that TEE (24h collection) was decreased in the FPh compared to the other phases. Again BMI or BW adjustments had no effect on the MC phase differences. Nevertheless, the estimated BMR (extrapolated from sleeping EE) was significantly higher in the LPh compared to the others.

Furthermore, Howe et al (1993) studied exercise EE and they found that when exercise was performed in the morning (10:30-11:00), it tended to induce a lower EE in the FPh than the LPh and MPh. This outcome was quite surprising as no differences were found when looking at exercise EE performed in the afternoon (15:00-15:30) or the total of both. Changes in substrate oxidation might have prompted the nearly significant differences in EE of morning exercise; however, the authors did not report respiratory exchange ratio (RER) of the exercise bouts. Yet, they found similar values for RER as a 24h measurement across the MC phases. Finally, they showed how P_4 had an impact on sleeping metabolic rate and 24h-EE while E_2 showed no association with these. Whether these findings are the appropriate reflection of each phase still remains uncertain as these values were taken solely on one day of each of the phases that had been selected according to the length of the MC of each participant.

In agreement with Howe et al's findings, Matsuo et al (1999) showed that EE was similar while cycling 60 min at a moderate exercise intensity (i.e. 60 % $\text{VO}_{2\text{max}}$) during the FPh and LPh in seven Japanese women, without differences in RER. However, they also observed that EE for the 6h following exercise was significantly increased in the LPh compared to the FPh with an RER being significantly lower in the LPh (i.e. there was a greater contribution of carbohydrates (CHO) to EE during the FPh than the LPh). Finally, RMR was also measured for 30 min in a fasted state before exercising on the testing day in each MC phase (designated by body temperature monitoring). Their results showed once again that RMR was significantly increased in the LPh compared to the FPh. Despite not measuring the ovarian hormones, the authors associated these differences in metabolism to the changes in oestrogen and P_4 levels throughout the MC. In fact, another study conducted by the same group of researchers showed that RMR and P_4 levels were significantly higher in the LPh than the FPh (Matsuo et al 1998).

To further support this notion, Day et al (2005) investigated whether inducing a decrease in oestrogens (E_1 and E_2) and P_4 levels by providing an antagonist of GnRH would cause a decrease in RMR. Their findings showed that when women exhibited significantly lower levels of oestrogens and P_4 as a response to the decreased FSH and LH, they also displayed a significantly lower RMR compared to the mid-LPh (7-9 days after ovulation) of a natural cycle. Moreover, they also showed a significant reduction in RMR in the MPh (days 2-6) compared to the LPh of the natural MC. It is worthwhile mentioning that the energy intake estimated by food diaries and the PA assessed by step counts during three days in each phase, was maintained during the different phases. Furthermore, their findings revealed that despite the differences in RMR, substrates metabolism at rest (i.e. RER) did not exhibit any changes by the decrease of the ovarian hormones. Despite showing strong evidence for how RMR can be affected by significant reductions in the ovarian hormones (induced by alterations in the sympathetic nervous system), this study did not examine the impact that high levels of oestrogens together with low concentrations in P_4 might have on RMR; this is of relevance as this occurs during the FPh.

However, not all studies have found differences in RMR throughout the MC. For example, an investigation in which 19 healthy, premenopausal naturally cycling (NC) women were monitored for RMR, BW and body composition for at least three days a week for a MC showed that there were no significant differences in RMR or anthropometric measurements between phases (Henry et al 2003). Nevertheless, this study revealed that the intra-individual CV for

RMR varied widely in women i.e. range of 1.7-10.4 %, therefore, it might be that RMR throughout phases of the MC was significantly modified in some but not all individuals. This agrees with Curtis et al (1996) who showed a CV% range 2.93-12.0% in a sample of 19 healthy women, although they did find a significantly greater BMR in the late LPh compared to the early FPh, in contrast to the Henry et al (2003) observations. Another study reporting the CV of RMR in 16 healthy Indian women, showed an average of 7.6% CV which is again higher than that observed in men (Piers et al 1995). However, likewise Henry et al (2003), they showed no differences between phases of the same MC in RMR, although Piers et al (1995) solely measured RMR twice in each phase.

Moreover, it needs to be noticed that in the Curtis et al (1996) and Henry et al (2003) studies the phases of the MC were estimated by self-reporting the starting and ending day of the cycle, thus the assignation of the phases might have been inaccurate and therefore the values appointed to each phase as well. Monitoring the phases of the MC by a reliable method, e.g. measuring the ovarian hormones, would have provided more persuasive results. Finally, it is worthwhile mentioning that Henry et al (2003) extended their study for a subsequent cycle in nine participants and found no significant differences in RMR CV% between MCs.

Considering the above studies and assuming that changing levels in the ovarian hormones throughout the MC induce alterations in energy metabolism in NC women, it seems rational to suggest that women taking HC may have a different energetic metabolism as the hormonal patterns are exogenously altered.

1.2.1.2. RMR in women using HC

Diffey and colleagues (1997) tested whether there were any variances in fasting BMR between 22 NC women and 24 women taking OC (OCW). Their results showed that there were no significant differences; however, when analysing the results taking into account BW or body composition (i.e. FFM + FM) OCW appeared to have ~ 5% significantly greater BMR than NC. Moreover, excluding eight participants who were unsure of the day of their MC and maintaining BW and body composition as covariates, the comparison between the two groups again showed a significantly higher BMR in OCW (n = 23) than in NC (n = 15). However, regarding the MC phase, there were similar values for BMR in the FPh (n = 18) and LPh (n = 20). Moreover, there was no interaction effect between OC and the MC phase despite the greater BMR observed in OCW. Nevertheless, these results would have been more persuasive if the

same participants were followed throughout the phases, rather than assessing BMR in only one phase as they do not take into account individual differences, therefore challenging any fluctuations throughout the phases of the MC within subjects.

A study that monitored RMR in five healthy women taking a monophasic combined OC ~5 days week for an entire MC showed no differences between phases nor between the days of taking the pill and the 7 days without the pill (Curtis, Henry and Ghusain-Choueiri 1996). Moreover, the range of the CV% was lower than the one observed in NC women (Curtis et al 1996). This would suggest a better maintenance of the energy balance throughout the MC and therefore a decreased risk on losing control on BW. Nevertheless, the sample size of the study was potentially not large enough to find possible differences.

Pelkman et al (2001) studied whether an injected progestin-only HC (Depo-Provera) could induce energy imbalances in a population of young healthy women who had not taken any HC during the last year. For this purpose, 20 women were randomly assigned in one of the following groups: placebo or Depo-Provera and their RMR and food intake was assessed before and after receiving the treatment. There were four testing sessions (one in the FPh and another in the LPh of two different cycles) before and after the intervention. Each testing session included the measurement of RMR on the first day and the measurement of 24h-food intake during three days. Their results showed that the Depo-Provera had no effect on food intake, macronutrient intake, BW and RMR. However, when examining the phases separately, there was a 2 % (30 kcal/d) increase post-treatment in RMR compared to the FPh. Conversely, no differences were observed compared to the LPh pre-treatment. Finally, it is worthwhile mentioning that data pre- treatment showed that energy intake and RMR were 4.3 % significantly greater in the LPh compared to the FPh. These results corresponded to 101 and 48 kcal/d increase in energy intake and RMR, respectively, which could ultimately explain the significant rise in BW in the LPh compared to the FPh (0.3 kg). Nevertheless, whether these small fluctuations in body balance might have an impact on BW management in the long-term in NC women, remains uncertain. Nonetheless, only the placebo group experienced a significant increase in BW (+ 0.7 kg), therefore, HC may facilitate BW management as they may provide a more stable RMR and a more controlled energy intake.

Caution is required when generalising this outcome as many different types of HC exist. Moreover, McNeill et al (1988) showed that averaged BMR significantly decreased (~170 kcal/d difference) in five young healthy women after taking an OC, causing an increase in BW (~0.8kg), albeit not significant. Although both studies were using a progesterone-only HC,

Pelkman et al (2001) employed an injected HC containing depot medroxyprogesterone acetate, whereas McNeill's HC was taken orally in a daily bases and was containing another progestin, i.e. levonorgestrel. However, a recent review looking at BW and the use of progesterone-only HC found little evidence on BW gain when comparing subtypes of progesterone-only HC (based on doses, regimens and formulations) or vs. non-using HC (Lopez et al 2011). Nevertheless, it was concluded that more controlled studies focusing on BW and HC should be conducted, thus, whether HC help or not on energy balance is still unknown.

In summary, RMR, the most important component of TEE, depends on several individual factors, e.g. age, sex, body composition, that have not been fully elucidated. Although many studies have aimed to understand whether the MC can also modify this part of the energy balance equation, it still remains ambiguous. Possible reasons behind the controversial results include incorrect assignation of the MC phase and taking measurements solely one day in the MC or of each phase which might not fully depict the whole story of what is happening.

1.2.2. Energy intake and appetite regulation

It is well known that multiple body regulators, e.g. brain, blood glucose levels, appetite hormones, gastric emptying velocity, control our food consumption in terms of what, when and how much do we eat (Coll et al 2007). However, other external aspects such as religion, food availability, hedonic eating and lifestyle can also determine human eating patterns (Näslund and Hellström 2013). In addition, women might have an added aspect which is the influence of the MC hormones, precisely, the effect of the ovarian hormones E_2 and P_4 . These fluctuate considerably throughout the MC and that might have a final effect on energy and macronutrient intake (Dye and Blundell 1997).

1.2.2.1. Central and peripheral regulation of food intake

It is well known that the hypothalamus is the part of the brain and the central nervous system (CNS) that is constantly receiving hormonal and chemical messages regarding the energy state of the organism to be able to maintain it in homeostasis (Schwartz et al 2000). In particular, the arcuate nucleus (ARC) along with the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA) and the ventromedial hypothalamic nucleus (VHN) are the areas of the hypothalamus that contain neurons that express the receptors for the hormonal and

metabolic factors e.g. leptin, ghrelin, that will induce an orexigenic or anorexigenic effect (i.e. inducing or inhibiting food intake, respectively) to our feeding system (Hirschberg 2012). The orexigenic neurons co-express the neuropeptide Y (NPY) and the agouti-related peptide (AgRP), whereas the anorexigenic neurons co-express cocaine-amphetamine regulated transcript (CART) and pro-opiomelanocortin (POMC) and its derivative α -melanocyte-stimulating hormone (α -MSH) (Schwartz et al 2000). Therefore, for example, during fasting, leptin levels are decreased and CART will be inhibited, whereas if leptin is injected, CART expression will be recovered (Gortari and Joseph-Bravo 2006).

Despite having what looks like a very controlled and autonomous feeding mechanism that should strictly respond to the physiological stimulus dictated by the peripheral hormones, there exist cortical and subcortical regions of the brain that regulate “hedonic eating” which can overwrite the hypothalamus commands. For instance, these areas (e.g. anterior cingulate, insula, amygdala, nucleus accumbens) can stimulate food consumption despite receiving a positive feedback of the energy status in the hypothalamus (Van Vugt 2010). This is possible due to the projections of the abovementioned neuropeptides into these areas of the brain, thus both areas together have an impact on EI. In fact, Frank et al (2010) demonstrated how different areas of the corticolimbic brain responded differently when high and low caloric foods are presented in front of women at the different phases of the MC. More specifically, they found that in the LPh certain reward areas of the brain (e.g. nucleus accumbens, amygdala, hippocampus) were only activated when participants were shown high-calorie foods, whereas in the FPh, the same brain areas were activated by high-, but also low-calorie foods. The authors related these changes to alterations in food preferences that could ultimately affect food intake. Furthermore, they suggested that the lack of activation in these areas of the brain might have responded to an impairment in the dopamine signalling similar to what is seen in obesity induced hyperphagia due to the modulatory action of the ovarian hormones on dopamine’s regulation.

As abovementioned, the changes in the expression of the orexigenic or anorexigenic neurons are dependent on the action of the peripheral hormones of which some will be outlined below:

Leptin is a cytokine synthesised by the adipose tissue that has been shown to suppress appetite and re-establish normal BW if injected in those who naturally cannot synthesise it because of a genetic mutation (Farooqi and O’Rahilly 2009). Its concentration in blood has been correlated with adiposity, therefore high concentrations are found in obese people

because of an induced-leptin resistance whereas low levels have been reported in patients with anorexia nervosa as an attempt of the body to stimulate food intake and achieve a healthy BW (Germain et al 2007). Interestingly a study in which obese subjects successfully achieved a reduction in their BW, showed that the provision of exogenous leptin could reverse the reduction in energy metabolism and satiety induced by the BW loss (Kissileff et al 2012). This suggests that the concomitant and disproportional decrease in EE and satiation induced by a reduction in BW is regulated by leptin. Therefore, leptin is regulated by the amount of fat stores and its main role in the short- and long-term energy regulation is to ensure the availability of sufficient energy stores that can guarantee the survival of the species (Rosenbaum and Leibel 2014). This is corroborated by the fact that low leptin levels cause a delay in the physical maturation during puberty in children and a paralysation of the reproductive system in women (i.e. MCs are anovulatory and amenorrheic), which both can be restored by the provision of exogenous leptin (Farooqi and O'Rahilly 2009, Mantzoros et al 2011). Furthermore, it has been shown that other organs such as the ovaries and the placenta, can also synthesise leptin (Margetic et al 2002).

Ghrelin is the only appetite-stimulating hormone currently known and this is secreted in the fundus of the stomach. Its concentration is abruptly increased before a meal whereas it is suppressed post food consumption (Yin et al 2009). Of note, if fasting for 24h its secretion pattern will be maintained around usual eating hours, i.e. ghrelin levels decrease after meal habitual times despite not having ingested any food (Natalucci et al 2005). Moreover, this hormone has the capacity of stimulating the release of the growth hormone (Messini et al 2013). Nevertheless, this will not occur in fasting conditions when the secretion pattern of the two hormones differs (Natalucci et al 2005). Decreased levels of ghrelin have been reported in obese humans (Tschop et al 2001), whereas elevated concentrations have been found in anorexia nervosa patients (Germain et al 2007). After normalizing BW ghrelin levels change to healthy levels. In addition, ghrelin presents different isoforms, acyl ghrelin (with an esterification with n-octanoic acid), des-acyl ghrelin (without the esterification) and obestatin. Initially, it was thought that the only active form was the acyl ghrelin, however, it has been recently shown that the dea-acyl form has also an important role interacting and counteracting the acyl form actions (Delhanty et al 2012).

Peptide tyrosine-tyrosine (PYY) is an appetite suppressor released from the L-cells of the gut that controls meal size and termination by suppressing gut motility (Onaga et al 2002). It has been found in two different forms: PYY₁₋₃₆ which is the main form at a fasted state and PYY₃₋₃₆

which is predominant after a meal (Grandt et al 1994). There has been found a negative correlation between body fat and PYY and its release after a meal is diminished in obese humans, however its administration exogenously can reduce food consumption in this particular population (Batterham et al 2003). Although the few studies that have looked at the impact of exogenous PYY on EI did not differentiate the effect between men and women, a study in female monkeys showed that, similarly to male monkeys, the injection of PYY in the lateral ventricle of the brain caused a suppression in EI that might be altered in the LPh of the MC (Papadimitriou et al 2007).

Glucagon-like peptide 1 (GLP-1) is another appetite suppressor synthesised by processing the proglucagon peptide in the L-cells of the gut. Its action is to inhibit food ingestion by reducing the gastric emptying rate of the stomach after a meal (Deane et al 2010). Moreover, it has the capacity of lowering blood glucose by increasing insulin secretion in a dose-manner response in healthy and type2 diabetics (Kjems et al 2003). Humans suffering from obesity or anorexia nervosa present low levels of GLP-I compared to normal weight counterparts (Germain et al 2007).

Cholecystokinin (CCK) is another gut hormone produced in the L-cells of the gut affecting the emptying rate of the stomach causing a reduction in food intake. Its actions comprise the contraction of the gall-bladder and the stimulation of pancreatic enzyme secretion (Hameed et al 2009). CCK levels increase 3-5-fold after the ingestion of a fat and protein rich meal (Crawley and Corwin 1994).

To summarise, appetite regulation conforms many different internal and external aspects to the individual that make difficult the prediction of the nature of his/her food intake.

1.2.2.2. Ovarian hormones and appetite regulation

Animal studies have shown cyclical changes in EI throughout the oestrus cycle in rodents and throughout the MC in primates; in particular, EI is reduced when high oestrogen levels are present, whereas it is increased when P_4 levels are high (Dye and Blundell 1997). Furthermore, the removal of their ovaries results in an increase in BW due to an increment in food intake and reduction in EE that can be reversed with the provision of oestrogens (Mauvais-Jarvis et al 2013).

Likewise, NC women undergo different eating patterns according to the MC phase. Many studies have shown that women have higher EI in the LPh compared to the FPh of the MC (Chung et al 2010, Johnson et al 1994, Li et al 1999, Lyons et al 1989); however, not all have found significant differences and this could be due in part to anovulatory cycles (Barr et al 1995) or unconfirmed ovulation (Bryant et al 2006). Nevertheless, the general consensus from different reviews is that food intake varies throughout the MC (Buffenstein et al 1995, Dye and Blundell 1997, McNeil and Doucet 2012).

In fact a recent review on the topic, showed that increases in EI during the LPh are of a magnitude of 87-500 kcal/d when comparing it to the FPh of the MC (McNeil and Doucet 2012). For example, Gil and coworkers (2009) showed a significant increase in EI (+ 2182 kJ/d), that was achieved through a higher intake of carbohydrates (CHO) (+ 67 g/d) and lipids (+ 55g/d) during the LPh compared to the FPh. Food diaries were collected during three days in each phase (two weekdays and one weekend day) and blood samples were taken on a single occasion in each phase to measure P_4 , oestrogen and leptin levels. As expected, P_4 and E_2 levels were significantly greater in the mid-LPh than in the FPh. Moreover, leptin levels also increased in LPh compared to FPh (~12% difference). This is of relevance as it suggests that not only the EI (and potentially appetite) fluctuates along with the ovarian steroids of the MC, but also leptin levels appear to be altered throughout the phases of the MC. In addition, this seemed to be the opposite of what would be expected, as above-mentioned, the provision of leptin causes a restriction in food consumption (Farooqi et al 2001).

Therefore, it could be suggested that during the LPh there might be a leptin resistance similar to what is observed in obese populations (Kolaczynski et al 1996) or rather, that leptin it is actually increased to comply its reproduction functions, e.g. prepare a women's body to become pregnant (Moschos et al 2002). Nonetheless, the only hormone that was significantly correlated with EI and CHO intake was P_4 . Riad-Gabriel et al (1998) also observed an increase in leptin levels in nine healthy NC women during the late FPh and the LPh compared to the early FPh, and this was in contrast to a lack of variation in post-menopausal women and men within 29 days.

Similar findings have been reported by Brennan et al (2009) who assessed food intake from a buffet 90 min after providing a load of 50g of glucose in 300ml of water to nine healthy women on three days of the MC: two in the FPh and one in the LPh. Their results indicated an increase in the amount of food and EI during LPh compared to FPh (~50 g and ~700 kJ difference, respectively). This was related to a faster emptying of the stomach, in particular, the time

needed for emptying 50% of the gastric glucose during LPh was 15 min less than during the FPh. This was corroborated by a higher post-meal release of GLP-1 during the LPh. Moreover, gastric emptying time was negatively correlated with P_4 levels which suggests that gastric emptying may be sensitive to the alterations in the ovarian steroids levels throughout the MC. However, it did not show any relation with E_2 concentration. Furthermore, it is worthwhile mentioning that due to the slower gastric emptying during the FPh, post-ingesting blood glucose and plasma insulin levels were also significantly lower than in the LPh, thus the glycaemia response was improved when P_4 was low in the FPh. Finally, CCK levels showed no differences during the 90min post- glucose ingestion despite the differences found in hunger and EI between phases. Nevertheless this was not entirely unexpected as CCK secretion seems to be more affected by fat and protein intake rather than glucose (Liddle et al 1985). Similarly, no differences were found at baseline.

Lundberg et al (2010) also found no differences in CCK levels either in plasma or in the cerebrospinal fluid when fasted. This contradicts with Frick et al (1990)'s findings showing a significant rise in basal CCK levels in the LPh compared to the FPh in eight NC women (5.2 ± 0.6 and 7.1 ± 0.9 pM, respectively). This is somewhat surprising considering that CCK reduces food intake. Thus, more studies are required to determine whether there exist changes in CCK levels at fasting levels and post-prandially across the MC.

Dafopoulos et al (2009) studied whether fasting ghrelin (acylated and unacylated), resistin and adiponectin levels varied significantly throughout the MC in eight healthy and NC women. Serum acylated and unacylated ghrelin were kept constantly at ~ 100 and 250 pg/ml, respectively during the MC. Likewise, adiponectin and resistin showed no significant changes. Nevertheless, E_2 and the two isoforms of ghrelin showed to be significantly correlated in some of the days of the MC (the authors did not indicate which days neither the strength of this correlation). Therefore, similarly to what has been shown in rodent studies, ghrelin could be at least partly regulated by E_2 , yet there is no evidence of such association in humans, thus how E_2 modulates food intake remains uncertain (Asarian and Geary 2013).

Pannacciulli et al (2006) reported that fasting GLP-1 levels were positively correlated with RMR (adjusted for sex, age, FFM and FM) in a population of men and women at their FPh. Therefore, it could be speculated that an increase in GLP-1 during the LPh observed by Brennan et al (2009) could partly explain the increase in RMR and food intake as an approach to maintain energy balance within the MC. Nevertheless, the differences between phases observed by Brennan et al (2009) were encountered after meal ingestion, whereas no changes

were found at fasting levels. Therefore, it would be interesting to study whether GLP-1 and RMR change simultaneously throughout the different phases of the MC. Pannacciulli et al (2006)'s results would have been more persuasive if reported separately the two sexes and in the different phases of the MC.

Along the same line of Pannacciulli et al, Guo et al (2006) studied how PYY levels might affect not only EI but also EE. In their study, 29 adults (21 men and eight women) were admitted into the metabolic ward to measure PYY, glucose and insulin concentrations in a fasted state, after a providing a standardized breakfast and every 30 min for the three following hours. On the day after, 23h-EE was measured in a calorimetric chamber and mean RMR was calculated. Their results showed that fasting PYY was negatively correlated to BMI, waist circumference, fasting triglyceride, postprandial insulin area under the curve (AUC) and 15h RMR. which might reveal a connection between this peptide and its role in BW regulation from the EE side of the energy balance equation. In fact, there was no significant correlation between the feelings of hunger and satiety with fasting PYY, nor postprandial PYY peak or PYY AUC except from latter one with satiety AUC. One of the drawbacks of this investigation was not separating the results based on participants' sex, despite controlling the MC by testing all women during their FPh. In fact, Hill et al (2011) studied PYY secretion pattern over 24-h in normal-women during the FPh and found that RMR was, on the contrary to Guo et al's outcome, positively correlated to fasting PYY, 24h-PYY AUC and 24h PYY levels (only the last two were significantly correlated). Although the discrepancies could be attributed to the inclusion criteria, both investigations ensured the potential effect of the MC, despite the fact that, to our knowledge, no studies have looked at fasting and postprandial PYY throughout the MC in humans and that no fluctuations have been observed in rhesus monkeys (Van Vugt 2010).

Therefore, it seems evident that there is a lack of knowledge in the area of appetite responses in fertile-aged women. This is somehow concerning since there are multiple studies investigating appetite hormones with the objective of having a better understanding of appetite regulation and aiming to find a strategy to target the current obesity epidemic. In fact, in the majority of studies, the MC phase and/or the use of HC is not even mentioned or taken into account (Karl et al 2013, Nieuwenhuizen et al 2009). When studies have attempted to control the potential effects that the phases of the MC might have on appetite and food regulation, women have been tested at the same MC phase (Guo et al 2006) or once they have reached menopause (Pasma et al 2008). Although these methods of controlling the potential influence of the fluctuations in the ovarian hormones within the MC are easy approaches to

manage this issue, it would not be appropriate to assume that the research outcome of that particular study is the real and complete picture of any possible effect (e.g. increased satiety, decreased food intake) a food/diet might have in this group of the population.

In summary, it is well known that numerous factors modify our food consumption during the day; however, how these respond and interact with the changes that oestrogen and P_4 experience during the MC is still uncertain. Future research should look at food intake and how this might be related to the appetite hormones in the different phases of the MC in NC women.

1.3. Summary and aims

To conclude, it seems plausible that the fluctuations experienced by the sex steroids, E_2 and P_4 , throughout the MC might have an effect on both sides of the energy balance equation. On the one hand, RMR seems to increase TEE in the LPh, and on the other hand, EI and appetite might be physiologically increased in response to this potential increase in energy demands in the LPh.

Therefore, this thesis aims to:

- Study the changes and the correlations between salivary and plasma E_2 and P_4 in the MC in a group of NC women and HC users.
- Investigate whether RMR is significantly increased in the LPh compared to the MPh and FPh in a group of NC women, while no changes are expected in HC users.
- Explore the association between RMR and the ovarian hormones in plasma and saliva as possible determiners of RMR.
- Investigate whether appetite responses (gastric emptying, PYY levels, appetite sensations and food intake) to the same breakfast vary according to the phase of the MC.

2. Methods

This chapter aimed to cover in depth the different methods used during the conduction of the three studies included in this thesis. Firstly, the common methods employed in all the studies (e.g. ethical approval, recruitment of participants) were explained and later, the specific techniques used in each study were also described. The specific protocols and statistical techniques used in each study were covered in the relevant chapters of the thesis.

2.1. Ethical approval

Prior to the start of all studies, ethical approval was applied for and obtained from the University Research Ethics Committee (UREC) at Oxford Brookes University (UREC Registration No: 130697 for Studies 1 and 2, and 140854 for Study 3 (Appendices). All participants were given full details of study protocols and the opportunity to ask questions. All participants gave written informed consent prior to participation (Appendices).

2.2. Participant recruitment and screening

Initially, the recruitment of participants was made through posters placed in Oxford Brookes University facilities e.g. library, sport centre, student accommodation, and also in local libraries or gyms (e.g. The Athlete Centre) as well as in the social media (e.g. Facebook). Previous experience within the Functional Food Centre at Oxford Brookes University had shown that advertising through posters can be ineffective in recruiting participants, therefore announcements in under-graduate and post-graduate lectures and informal recruitment through personal networks was also used. Moreover, the study was advertised in the Oxford Brookes University Research Activity Group, on the Functional Food Centre website and in the volunteers section of a local website (<http://www.dailyinfo.co.uk/>).

Interested participants were provided with further information on the study in the form of an information sheet (Appendices), sent by email. After at least 24 hours of having read the information sheet participants came to the Functional Food Centre to discuss any queries they had with the researcher and to sign the consent form. Finally, a screening questionnaire was completed to ensure eligibility of the participant in the study.

During the screening session, participants completed a questionnaire (Appendices) to verify that they were healthy women of 18-40 years of age, with regular menstrual cycles (i.e. cycle length of 25-35 days) and not lactating or pregnant for the previous six months at least. Participants were excluded if their BMI $>30 \text{ kg/m}^2$ or they were suffering from any metabolic/genetic diseases or taking any medications known to interfere with metabolism, e.g. hypothyroidism. Finally, those who showed to be restrictive eaters were also excluded. This was assessed by the combination of two adapted restrictive eating questionnaires: the Dutch Eating Behaviour Questionnaire (DEBQ) (van Strien et al 1986) and the Three-factor eating questionnaire – restraint eating (TFEQ) (Stunkard and Messick 1985) (Appendices). Participants with a TFEQ score of >10 and a DEBQ >2.5 were considered restrictive eaters and therefore they were excluded from participating in the study.

In addition to the above general inclusion/exclusion criteria, in Study 3 HC users were not eligible to participate because the aim of the investigation was to look at the appetite responses of women whose sex hormones fluctuate naturally over the course of a MC. Moreover, participants suffering from anaemia were excluded from participating in the second study due to any potential harm caused by the increased amount of blood drawn throughout the MC. In addition, participants who had an allergy/intolerance to any of the foods given in the study, did not consume breakfast and lunch habitually or were attempting to lose weight were also excluded. Finally, smokers and participants with a disease (e.g. Gilbert's syndrome) or taking medication known to interfere with appetite (e.g. codeine) were excluded from the study.

At the end of each study, participants were compensated with a £50 Amazon voucher as well as a full report detailing their personal results including their individual RMR, menstrual cycle profile, body composition, PA and eating patterns, depending on the study they participated in.

2.3. Baseline characteristics

2.3.1. Lifestyle questionnaire

During the first visit to the Functional Food Centre, participants completed a lifestyle questionnaire that encompassed the Physical Activity Questionnaire (PAQ) (Baecke et al 1982) and the Premenstrual Syndrome Screening Tool (PSST) (Steiner et al 2003) to have a better

characterisation of population being studied. The PAQ (Appendices) consists of three different indexes, i.e. work, sport and leisure that were summed and to obtain an overall PA index (PAI) with higher scores indicating greater PA levels. The PSST (Appendices) is a validated screening tool that can aid in the diagnosis of the premenstrual syndrome (PMS) and the premenstrual dysphoric disorder (PMDD) by rating the premenstrual symptoms in a four-levels scale i.e. '*not at all*', '*mild*', '*moderate*' and '*severe*' (Steiner et al 2003).

2.3.2. Body composition assessment

All anthropometric measurements were made in the fasting state. Height was measured to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK), with subjects standing upright and without shoes. BW (to the nearest 0.1 kg) and body composition, i.e. FM, FFM and body water (BW), were measured with the Tanita Body Composition Analyzer BC-418MA (Tanita Ltd, West Drayton, UK). Finally, waist and hip circumferences were measured to the nearest 0.1cm with an anthropometric tape (Lufkin W606PM flexible steel tape, Maryland, USA). These were determined as the narrowest and widest circumference of the waist and hip, respectively (Stewart et al 2011).

2.4. Blood sample collection and analyses

2.4.1. Blood collection and storage

Blood samples were collected from a large vein of the anti-cubital fosse of the arm of the participants with K2E-EDTA tubes (BD Vacutainer, Becton Dickinson, UK) by a trained phlebotomist. When only one blood sample was required during the visit to the lab (studies 1, and 2) this was withdrawn into a 6 ml tube via venepuncture using a 21G multi sample needle (BD Vacutainer, PrecisionGlide). In Study 3, repeated blood sampling during the same visit was required, thus plasma samples were obtained from an inserted cannula (BD Venflon Pro Safety 20GA, Becton Dickinson Induction Therapy, Singapore). The cannulation technique involves flushing 3ml of 0.9% sodium chloride into the system with a needle-free syringe before and after collecting each sample to ensure that the cannula is still patent and to remove any blood from the cannula to avoid clotting. Before disconnecting the syringe to collect the blood, a 3 ml sample was withdrawn with the same syringe to ensure the sample was clean from sodium chloride (Fig. 2.1). A 4ml blood sample was withdrawn from the cannula for every time point,

except for the baseline when 8ml were collected to measure the ovarian hormones. After collection, blood samples were kept in ice until they were centrifuged at 4°C for 10 minutes at 4000 rpm (MC-6, Sarstedt Ltd, Leicester, UK) to extract the plasma. This was then frozen at -80°C in different aliquots until analysis.

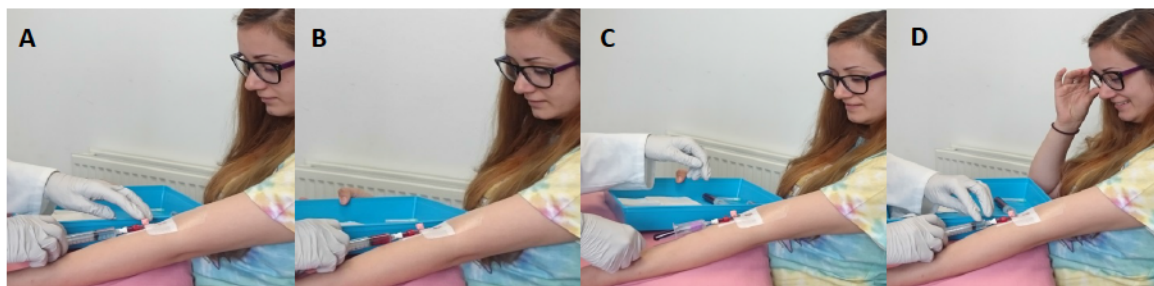


Fig 2.1. Collection of a blood sample from a cannula: **(A)** Flushing sodium chloride into the system with to ensure patency of the cannula **(B)** Extraction of a sample with the syringe to ensure the collection of a blood sample clean of sodium chloride **(C)** Extraction of the blood sample with a tube **(D)** Flushing into the system to maintain the cannula patent until the collection of the subsequent sample.

2.4.2. Plasma E_2 and P_4 measurement (Study 1, 2 and 4)

Plasma samples were thawed to measure E_2 and P_4 by an ElectroChemiluminescence immunoassay (ECLIA) with a Cobas e411 semi-automated analyser (Roche diagnostics Burgess Hill, UK). The ECLIA employs the competitive test principle to quantify the concentrations of E_2 and P_4 in plasma (Roche 2009a, b). In preparation for the analyses, all reagents and samples were brought to room temperature. Firstly, the reagent kits were calibrated using their respective CalSets. Once plasma samples were completely thawed, these were vortexed and centrifuged at 4000 rpm for one minute. Then, 300 μ l of plasma was added into a sample cup in order to be analysed by the semi-automated analyser. The analyser executed two incubations: (1) the sample was incubated with the specific biotinylated antibodies for E_2 and P_4 and also E_2/P_4 labelled with a ruthenium complex (Tris(2,2'-bipyridyl)ruthenium(II)) was added; (2) streptavidin was added to interact with the biotin of the antibodies. The amount of labelled antigen was inversely proportional to the concentrations of E_2/P_4 of the plasma samples. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured into the surface of the electrode. Unbound particles were washed out and a voltage was applied to the electrode which induced a chemiluminescent emission that was measured by a photomultiplier. Results were automatically determined with a calibration curve (Roche 2009a, b).

2.4.3. PYY (total) measurement (Study 3)

Plasma total PYY (both 1-36 and 3-36) concentrations were measured using a direct sandwich enzyme-linked-immunosorbent assay (ELISA) kit (EMD Millipore). The testing principal of this assay is to measure the levels of total PYY in plasma by capturing the antigen with two different antibodies (the *capture* and the *detection* antibodies) that bind to two different epitopes of the PYY molecule (EMD Millipore Corporation 2014). The capture antibody is the one coating the microtiter plate that will bind to the PYY of the plasma samples and the detection antibody is the one measuring the concentrations of antigen by being conjugated to an enzyme (R&D Systems (n.d.)). This enzyme will bind to a detection reagent that reacts with a substrate solution to develop colour and its optical density will be used to measure the levels of PYY in each sample. Since the increase in absorbency is directly proportional to the amount of PYY present in the samples, the latter can be delivered by interpolation from the reference curve generated by the standards containing known PYY concentrations.

Assay protocol was executed following manufacturer's instructions (EMD Millipore Corporation 2014). The layout of the wells of the plate was designed with duplicates of unknowns (UNK) i.e. plasma sample, standards (ST), quality controls (QC), and blanks, as shown in Fig 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNK_1	UNK_1	UNK_9	UNK_9	UNK_17	UNK_17	UNK_25	UNK_25	UNK_33	UNK_33	ST4	ST4
B	UNK_2	UNK_2	UNK_10	UNK_10	UNK_18	UNK_18	UNK_26	UNK_26	UNK_34	UNK_34	ST5	ST5
C	UNK_3	UNK_3	UNK_11	UNK_11	UNK_19	UNK_19	UNK_27	UNK_27	UNK_35	UNK_35	ST6	ST6
D	UNK_4	UNK_4	UNK_12	UNK_12	UNK_20	UNK_20	UNK_28	UNK_28	UNK_36	UNK_36	ST7	ST7
E	UNK_5	UNK_5	UNK_13	UNK_13	UNK_21	UNK_21	UNK_29	UNK_29	UNK_37	UNK_37	ST8	ST8
F	UNK_6	UNK_6	UNK_14	UNK_14	UNK_22	UNK_22	UNK_30	UNK_30	ST1	ST1	QC1	QC1
G	UNK_7	UNK_7	UNK_15	UNK_15	UNK_23	UNK_23	UNK_31	UNK_31	ST2	ST2	QC2	QC2
H	UNK_8	UNK_8	UNK_16	UNK_16	UNK_24	UNK_24	UNK_32	UNK_32	ST3	ST3	Blank	Blank

Fig 2.2. Plate layout for PYY assay

For the preparation of the assays, all reagents and samples were brought to room temperature and once plasma samples were completely thawed, these were vortexed and centrifuged at 3000 rpm for 1 minute. Before proceeding with the assay, preparation of the STs and the QCs was required. First, the vials containing PYY standard and the two QCs (their concentration was dependent and indicated on each individual lot) were mixed with 500 μ l deionized water each and left to sit for ~5 minutes to completely dissolve. Six tubes were labelled (ST1-6) and 200 μ l assay buffer (borate saline) was added in each tube. Then 200 μ l reconstituted PYY standard

was added into PYY ST1 to be serially diluted two-fold by pipetting 200 μ l from tube 1 to tube 2, from tube 2 to tube 3 and so on until tube 6 was made up with a final concentration of 64-fold less than the reconstituted PYY standard (Fig 2.3). Before aspirating from one standard, the tube was mixed well and a new pipette tip was used to pipette to the next tube.

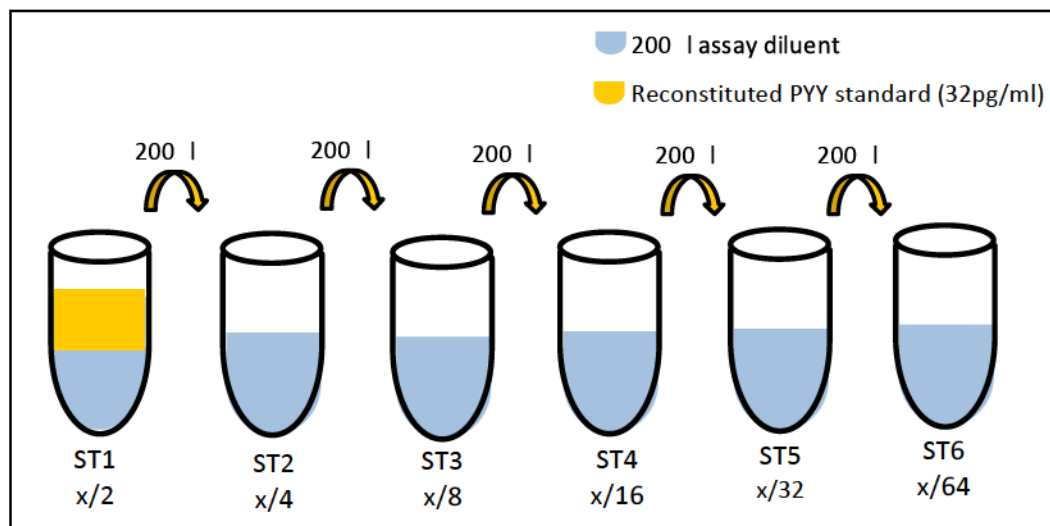


Fig 2.3. Serial dilution for PYY standards

The first step was to add 300 μ l diluted wash buffer (buffered saline) into each well. This was decanted and tapped into absorbent paper towels three times and 20 μ l matrix solution (contains DPP IV inhibitor) was added into the blank, STs and QC wells. Subsequently, 20 μ l plasma, ST, QC, and assay buffer (in the blank wells) was added in the designated wells in duplicate. Finally, 20 μ l blocking solution was added into each well and left to incubate for 30 minutes at room temperature in a plate shaker (MicroPlate Shaker PMS-1000i, Grant bio, Grant Instruments, Cambridge, UK) at 450 rpm.

Following this, 50 μ l mixture of the capture (rabbit antihuman PYY) and detection (biotinylated antihuman PYY) antibodies was added into each well and the plate was left to incubate at room temperature for 1.5 hours to enable the PYY of the samples to bind to the antibodies. All the unbound particles were washed away by decanting plus tapping the plate three times as previously done. Subsequently, 100 μ l enzyme solution (Streptavidin-HRP Conjugate in Buffer) was added into each well to bind to the biotin of the detection antibody while shaking at 400 rpm during 30 minutes at room temperature. Residual liquid was decanted from the plate and this was washed six times as previously done. Then, 100 μ l of TMB substrate solution was added into each well to react with the HRP bound to the detection antibody which produced a blue colour while the plate was shaken for 5-20 minutes at 450 rpm. Finally, 100 μ l stop

solution containing sulphuric acid was added to end the colour development and the plate was shaken by hand until the wells turned into a yellow colour.

Within five minutes, the plate was read in a plate reader (LT-4500 Microplate ELISA Reader, Labtech International Ltd, UK) at a wavelength of 450 nm and 590 nm to obtain two different optical densities (OD) of all the wells. The two readings were subtracted and the an online software (My Assays Ltd) computed the concentrations of total PYY of each plasma sample using an equation derived from a 4-parameter logistic equation curve obtained by plotting PYY levels of the standards on the x-axis (pg/ml) and their absorbance (nm) y-axis (Fig 2.4).

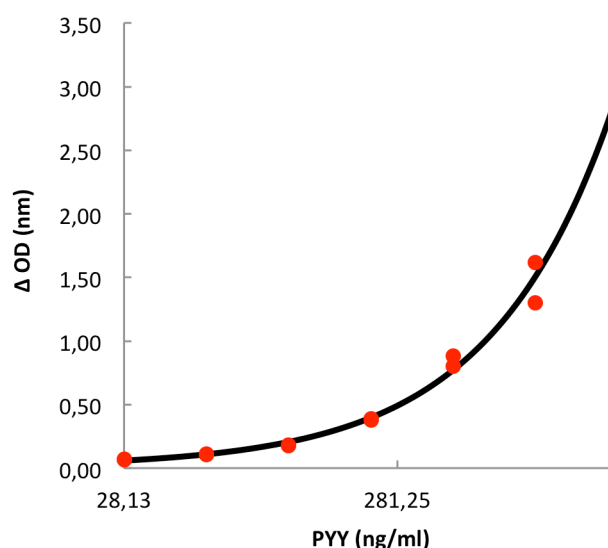


Fig 2.4. Example PYY 4parameter curve fit

The assay was accepted if one of the two QC was $\pm 2SD$ of the applicable mean of the range given by the supplier. Average concentrations between duplicates were accepted provided that the CV% between duplicates was $<15\%$. The minimal concentration of PYY that can be distinguished from 0 is 6.5pg/ml. To minimize error within participant, each participant had their PYY of the three visits measured in the same microplate.

Concentrations of PYY were used to calculate the total area under the curve (AUC) using the trapezoidal method at min 60, 120, 180, 210 and 240 from baseline (before breakfast). When time point samples were not available due to collection procedure issues, an average between the previous and subsequent collection time points was made to estimate the concentration of the missing sample a an obtain a representative PYY AUC.

2.5. Saliva sample collection and analyses (Study 1)

2.5.1. Saliva collection and storage

Saliva samples were collected using the unstimulated passive drool technique (Salimetrics 2008) under standardised conditions: early in the morning, in a fasted state without having consumed alcohol for the previous 12 hours and after rinsing thoroughly the mouth with water 10 minutes before a ~2 ml sample was collected. Participants were asked to allow the saliva to pool in their mouth and then with the head tilted forward they self-collected a saliva sample in a polypropylene tube (Sarstedt, Nümbrecht, Germany). Participants were instructed to imagine eating their favourite food to stimulate saliva secretion. Samples were kept in ice until they were distributed in different aliquots to be stored at -40°C within four hours of collection.

2.5.2. Salivary E₂ and P₄ measurement

Free E₂ and P₄ concentrations in saliva were measured by an EIA kit (Salimetrics). The test principle of these assays is a competitive EIA in which the antigen (i.e. E₂/P₄) of the saliva sample competes against a reagent containing enzyme-conjugated E₂/P₄ to bind to the antigen antibody. The more enzyme-conjugated E₂/P₄ bound to the antibodies, the less antigen is present in our saliva samples and the more colour is produced by the enzyme-conjugated E₂/P₄, i.e. the optical density/colour of the sample is inversely proportional to the amount of E₂/P₄ in the sample (Sino Biological Inc. (n.d.)).

Assay protocols were performed following manufacturer's instructions (Salimetrics 2012a, b). The layout of the wells of the plate with unknowns (UNK) i.e. saliva sample, standards (ST), quality controls (QC) high and low, zeros and non-specific binding (NSB) wells (i.e. wells without anti-E₂ antibody) was designed as shown in Fig. 2.5. For the preparation of the assays, all reagents and samples were brought to room temperature and, once saliva samples were completely thawed, these were vortexed and centrifuged at 3000 rpm for 15 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNK_1	UNK_1	UNK_9	UNK_9	UNK_17	UNK_17	UNK_25	UNK_25	UNK_33	UNK_33	ST1	ST1
B	UNK_2	UNK_2	UNK_10	UNK_10	UNK_18	UNK_18	UNK_26	UNK_26	UNK_34	UNK_34	ST2	ST2
C	UNK_3	UNK_3	UNK_11	UNK_11	UNK_19	UNK_19	UNK_27	UNK_27	UNK_35	UNK_35	ST3	ST3
D	UNK_4	UNK_4	UNK_12	UNK_12	UNK_20	UNK_20	UNK_28	UNK_28	UNK_36	UNK_36	ST4	ST4
E	UNK_5	UNK_5	UNK_13	UNK_13	UNK_21	UNK_21	UNK_29	UNK_29	UNK_37	UNK_37	ST5	ST5
F	UNK_6	UNK_6	UNK_14	UNK_14	UNK_22	UNK_22	UNK_30	UNK_30	UNK_38	UNK_38	ST6	ST6
G	UNK_7	UNK_7	UNK_15	UNK_15	UNK_23	UNK_23	UNK_31	UNK_31	Zero	Zero	Low	Low
H	UNK_8	UNK_8	UNK_16	UNK_16	UNK_24	UNK_24	UNK_32	UNK_32	NSB	NSB	High	High

Fig 2.5. Plate layout for saliva E₂ and P₄ assays

2.5.2.1. Salivary E₂ EIA protocol

The first step was the preparation of the STs. Five tubes were labelled corresponding to STs 2 to 6 in which 300 μ l E₂ assay diluent was added. Then E₂ ST1 (32 pg/ml) was serially diluted 2 fold by pipetting 300 μ l from tube 1 to tube 2, from tube 2 to tube 3 and so on until tube 6 was made up with a final concentration of 1 pg/ml (Fig 2.6). (Before aspirating from one standard, the tube was mixed well and a new pipette tip was used to pipette to the next tube).

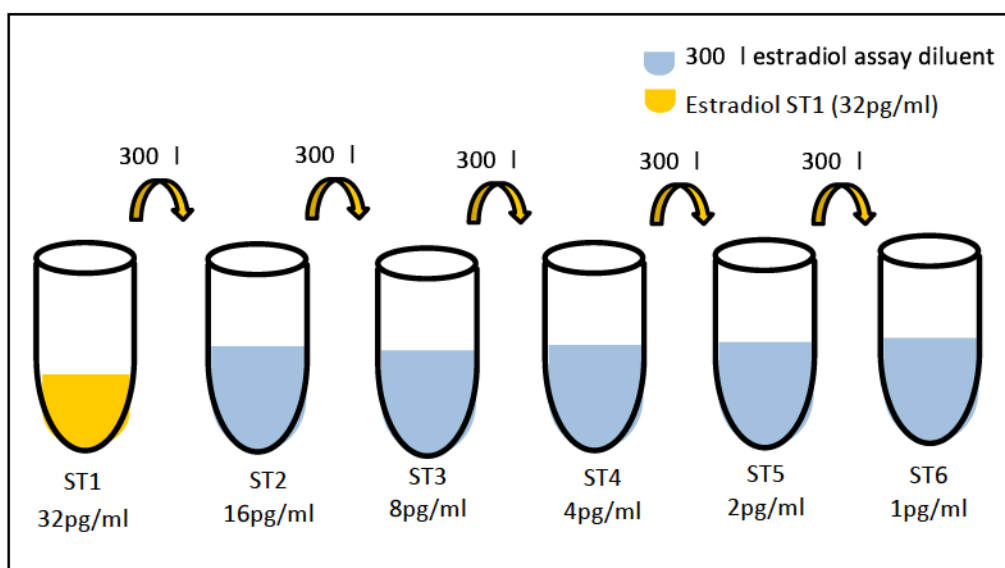


Fig 2.6. Serial dilution for E₂ standards

Following this, 100 μ l each ST, UNK and QC was pipetted into the corresponding wells and 100 μ l E₂ assay diluent was pipetted into the zero and the NSB wells of the plate coated with rabbit antibodies for E₂. Subsequently, the HRP-conjugated E₂ was diluted 1:800 by adding 15 μ l into 12 ml of E₂ assay diluent. This solution was mixed and 100 μ l was added into each well using a multichannel pipette. The plate was covered and left to incubate at room temperature in a plate shaker for 5 minutes and then 115 minutes more out of the plate shaker. This allowed the E₂ from each saliva sample to compete against the HRP-conjugated E₂ to bind to the antibodies of the plate. Once the incubation was completed, the plate was washed out to remove any unbound components by adding 300 μ l pre-diluted wash buffer containing phosphate into each well with a multichannel pipette. The plate was decanted into a sink to finally be blotted onto paper towels to ensure there was no wash left in the wells. The washing process was repeated three times to ensure complete wash out of unbound particles and then 200 μ l TMB substrate solution was added into each of the wells which reacted with the HRP of the E₂ conjugated producing a blue colour while the plate was being shaken for five minutes at 500 rpm. Subsequently, the plate was incubated in the dark at room temperature for 25 minutes to further allow the colour reaction to occur and then 50 μ l stop solution containing

sulphuric acid was added to end the colour development. The plate was shaken at 500 rpm for three minutes so that the wells turned into a yellow colour and then the plate was read in a plate reader at a wavelength of 450 nm within 10 minutes of adding the stop solution to obtain the OD of all the wells.

The plate reader software (Gen5, BioTek Instruments, Inc) calculated the concentrations of E_2 of each saliva sample using an equation derived from a 4-parameter non-linear regression curve obtained by plotting E_2 levels of the standards on the x-axis and their percentage of bound E_2 in the y-axis (Fig. 2.7).

Percentage of bound E_2 of each duplicate of standards, saliva samples, zeros and controls were calculated as follows:

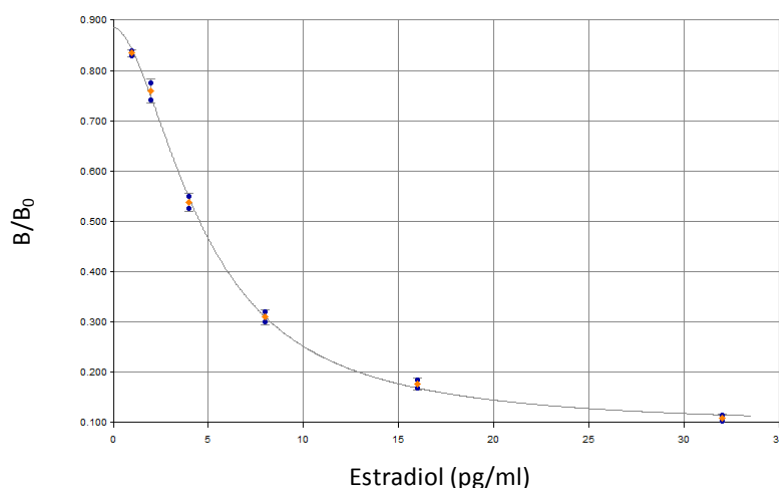


Fig 2.7. Example estradiol 4-parameter curve fit

High and low QC were checked to be within limits and average concentrations between duplicates were accepted provided that the CV% between duplicates was <15%. The minimal concentration of E_2 that can be distinguished from 0 is 0.1 pg/ml.

2.5.2.2. Salivary P_4 EIA protocol

The measurement of P_4 in saliva by EIA followed the same protocol as the salivary E_2 with some obvious changes in the reagents as well as the quantities of some.

In preparation of the STs, five tubes were labelled corresponding to STs 2 to 6 in which 200 μ l of P_4 assay diluent was added. P_4 ST1 (2430pg/ml) was serially diluted three-fold by pipetting 200 μ l from tube 1 to tube 2, from tube 2 to tube 3 and so on until tube 6 was made up with a final concentration of 10 pg/ml (Fig. 2.8). (Before aspirating from one standard, the tube was mixed well and a new pipette tip was used to pipette to the next tube).

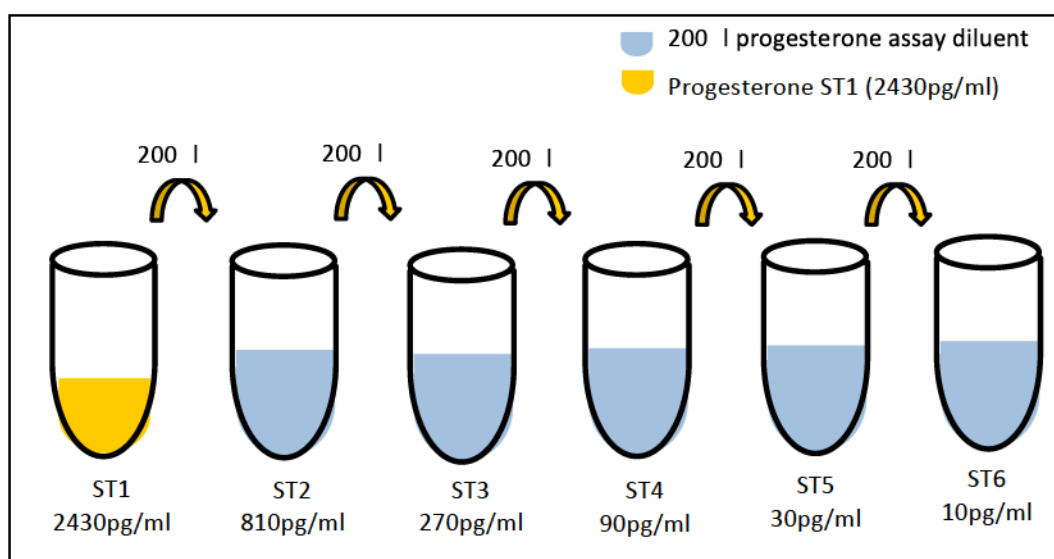


Fig 2.8. Serial dilution for P_4 standards

Following this, 50 μ l ST, UNK and QC were pipetted into the corresponding wells and 50 μ l P_4 assay diluent were pipetted into the zero and the NSB wells of the plate coated with rabbit antibodies for P_4 . Subsequently, the HRP-conjugated P_4 was diluted 1:800 by adding 22.5 μ l into 18 ml P_4 assay diluent. This solution was mixed and 150 μ l was added into each well using a multichannel pipette. The plate was covered and left to incubate at room temperature while shaking in a plate shaker 60 minutes. This allowed the P_4 from each saliva sample to compete against the HRP-conjugated P_4 to bind to the antibodies of the plate. Once the incubation was complete, the plate was washed out to remove any unbound components by adding 300 μ l pre-diluted wash buffer containing phosphate into each well with a multichannel pipette. Then the plate was decanted into a sink to finally be blotted onto paper towels to ensure there was no wash left in the wells. The washing process was repeated three times and then 200 μ l TMB substrate solution was added into each of the wells. This reacted with the HRP of the P_4 conjugated producing a blue colour while the plate was being shaken for five minutes at 500

rpm. Subsequently, the plate was incubated in the dark at room temperature for 25 minutes to further allow the colour reaction to occur and then 50 μ l stop solution containing sulphuric acid was added to end the colour development. The plate was shaken at 500 rpm for three minutes so that the wells turned into a yellow colour. Finally, the plate was read in a plate reader at a wavelength of 450 nm within 10 minutes of adding the stop solution to obtain OD of all the wells.

Once more, P_4 concentrations of each saliva sample were calculated using the equation of the 4-parameter non-linear regression curve obtained by plotting P_4 levels of the standards on the x-axis and their percentage of bound P_4 in the y-axis (Fig. 2.9). Percentage of bound P_4 of each duplicate of standards, saliva samples, zeros and controls were calculated as abovementioned in the EIA protocol.

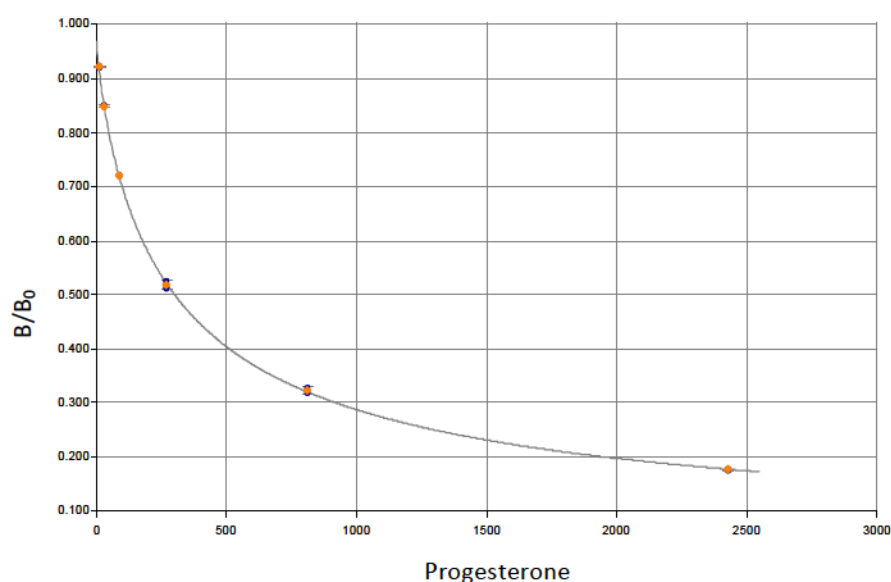


Fig 2.9. Example progesterone 4-parameter curve fit

High and low QC were checked to be within limits and average concentrations between duplicates were accepted provided that the CV% between duplicates was <15%. The minimal concentration of P_4 that can be distinguished from 0 is 5 pg/ml.

2.6. RMR and substrate metabolism assessment (Study 2)

2.6.1. RMR measurement

RMR was measured by the hooded open circuit indirect calorimeter GEM (GEMNutrition Ltd, Daresbury, UK). Previous research in our laboratory has shown that the GEMNutrition is a reliable tool for repeated measures of RMR (CV% <5%) (Kennedy et al 2014). The testing principle of any indirect calorimeter is that by measuring the volumes of oxygen consumption (VO_2) (ml/min) and the carbon dioxide production (VCO_2) (ml/min) from breath samples, the heat (energy) produced by the metabolic oxidative processes can be calculated (in kcal/d). To facilitate this, the GEM applies the adapted version of the Weir equation in which protein oxidation is considered negligible (Weir 1949):

$$\text{RMR (kcal/d)} = (3.9 \text{VO}_2 + 1.1 \text{VCO}_2) \times 1.44$$

Where 1.44 computes the kcal requirements per day.

On the testing day participants attended the laboratory in the morning (between 07:00-10:00) after a 10-12 hour overnight fast and rested for 30 minutes to achieve a state of stabilization after their commute to the laboratory. RMR was measured with the GEM for 30 minutes under the following standardized conditions (Compher et al 2006) (Fig. 2.10): lying down in a supine position in a thermoneutral environment (20–25°C) and without having practised any strenuous or non-habitual physical exercise as well as not having consumed alcohol or caffeine the evening before. The first five minutes of measurement were automatically discarded so



Fig 10. Measurement of RMR. **(A)** First 30 min lying down for stabilisation **(B)** Last 30 min under the canopy to measure RMR

that the participants had enough time to adapt to the hood. Moreover, of the remaining 25 minutes measurement, the first five minutes were used to ascertain that the participant was stable by achieving $\leq 10\%$ CV for VO_2 and VCO_2 (Compher et al 2006). Thus, the average of the respiratory gas exchange data of the 25 minutes measurement was used to calculate the RMR

after applying the modified Weir equation. When the % CV for VO_2 and VCO_2 was indicating the participant was unstable, that day was removed from the averaged RMR measurements of the cycle and phase of that participant. RMR was calculated as absolute RMR (i.e. per day) and relative RMR (i.e. per kg of BW). During the entire time lying down (resting or under the canopy), participants were allowed to read, watch films or listen to music while being quiet. These conditions were kept constant between visits within participants.

The calorimeter was calibrated before every measurement with two gas bottles (1% CO_2 /20% O_2/N_2 and Nitrogen Zero grade) and alcohol checks were performed monthly for further calibration.

2.6.2. RMR measured vs. RMR predicted

Averaged measured RMR (RMR_m) of the entire MC and per phase were compared to predicted RMR (RMR_p) from ten different equations valid for adult females according to the latest published scientific opinion on dietary reference values for energy by the European Food Safety Authority (EFSA 2013) (Table 2.1). Bias was determined as the mean percentage difference between RMR_m and RMR_p and accuracy as the percentage of participants with an RMR_p within $\pm 10\%$ of RMR_m.

2.6.3. Substrates metabolism measurement

From the volumes of O_2 consumed and CO_2 produced (l/min), oxidation rates of the substrates were calculated using the adapted Frayn stoichiometric equations where negligible protein oxidation is assumed (Frayn 1983):

$$\text{Fat oxidation rates } (g/min) = 1.67 \times VO_2 - 1.67 \times VCO_2$$

$$\text{Carbohydrate oxidation rates } (g/min) = 4.55 \times VO_2 - 3.21 \times VCO_2$$

The average rate during the 25 minutes measurement was used as the substrate oxidation value of the visit. Moreover, each substrate was converted to kcal/min using the Atwater Factors (1g fat = 9kcal and 1g carbohydrates = 4kcal) (Feher 2012) to further calculate each of the substrate's contribution as a percentage of the total energy oxidised.

Table 2.1. Validated predictive equations for female adults (EFSA 2013)

	Predictive equation	Units
(1) Harris Benedict	$BM \times 9.5634 + HT(cm) \times 1.8496 - Age \times 4.6756 + 655.0955$	(kcal/d)
(2) Schofield_1	Age 18 – 30 y: $0.062 \times BM + 2.036$ Age 30 - 60 y: $0.034 \times BM + 3.538$	(MJ/d)
(3) Schofield_2	Age 18 – 30 y: $0.057 \times BW + 1.184 \times HT(m) + 0.411$ Age 30 - 60 y: $0.034 \times BM + 0.006 \times HT(m) + 3.53$	(MJ/d)
(4) Mifflin	$9.99 \times BM + 6.25 \times HT(cm) - 4.92 \times Age - 161$	(kcal/d)
(5) Muller_1	$0.47 \times BM - 0.01452 \times Age + 1.009 \times Sex + 3.21$	(MJ/d)
(6) Muller_2	BMI > 18.5 to 25: $0.02219 \times BM + 0.02118 \times HT(cm) - 0.01191 \times Age + 0.884 \times Sex + 1.233$ BMI > 25 to <30: $0.04507 \times BM - 0.01553 \times Age + 1.006 \times Sex + 3.407$ BMI >30: $0.05 \times BM - 0.0158 \times Age + 1.103 \times Sex + 2.924$	(MJ/d)
(7) Muller_3	$0.5192 \times FM + 0.04036 \times FM + 0.869 \times Sex - 0.01181 \times Age + 2.992$	(MJ/d)
(8) Muller_4	BMI > 18.5 to 25: $0.0455 \times FFM + 0.0278 \times FM + 0.879 \times Sex - 0.0129 \times Age + 3.634$ BMI > 25 to <30: $0.03776 \times FFM + 0.03013 \times FM + 0.93 \times Sex - 0.01196 \times Age + 3.928$ BMI > 30: $0.05685 \times FFM + 0.04022 \times FM + 0.808 \times Sex - 0.01402 \times Age + 2.818$	(MJ/d)
(9) Henry_1	Age 18-30 y: $0.0546 \times BW + 2.33$ Age 30-60 y: $0.0407 \times BW + 2.90$	(MJ/d)
(10) Henry_2	Age 18-30 y: $0.0433 \times BM + 2.57 \times HT(m) - 1.18$ Age 30-60 y: $0.0342 \times BM + 2.10 \times HT(m) - 0.0486$	(MJ/d)

Where *BW* = body weight (kg); *HT* = height; *FM* = fat mass (kg); *FFM* = fat free mass (kg); *Sex* = 0.

2.7. Gastric emptying assessment (Study 3)

2.7.1. Breakfast characteristics

In Study 3, potential changes in the gastric emptying (GE) throughout the MC were examined after consuming the same breakfast on a single day in each of the three phases of the MC.

A breakfast containing scrambled eggs on toast, fruit (pineapple) and a drink (i.e. coffee, tea or water) was given to participants between 07:30-09:30. The breakfast was standardised amongst participants and provided 375-395 kcal of which 35%, 38% and 23% were in the form of fat, carbohydrates and protein, respectively. The energy provided by the breakfast accounted for 17-18 % of the total daily energy requirements for an average woman (19-44 years) with median PA level of 1.63 i.e. 2103-2175 kcal/d (SACN 2011). The nutritional composition of the breakfast is shown in Table 2.2.

The preparation of the breakfast was as following:

- Two slices of frozen wholemeal bread (Tesco Everyday Value Medium Sliced Wholemeal Bread) were defrosted at room temperature and then grilled for three minutes on each side and spread with 15 g olive spread (Tesco).
- For the scrambled eggs, two free-range eggs were beaten and 100 µg ¹³C octanoic acid (see section 2.7.3) was added for the measurement of gastric emptying. The eggs were cooked in the microwave for 30s + 30s + 15s + 15s while stirring between bouts.
- Drained pineapple chunks (Tesco) were weighed into a bowl.
- The drink was chosen by each participant with the aim of replicating their usual breakfast. This could be tea, coffee or water with/out milk and sugar. The total amount of liquid was 250 ml, thus if taking coffee (2g of Nescafe Original Instant Coffee) or tea (1 bag of Yorkshire Tea) with milk (British semi skimmed milk, Tesco), the hot beverages were prepared with 210 ml of hot water plus 40 g of milk. If the participant wanted sugar, on the first visit she served herself *ad libitum* from a pre-weighed bowl and then the amount was replicated for the next two visits.

Participants were asked to finish the whole breakfast within 15 minutes. After finishing their breakfast, participants were instructed not to eat or drink anything else until lunch with the exception of a 330 ml bottle of water provided by the researcher. Water consumption was calculated by weighing the bottle before and after; the same amount was replicated on the subsequent visits.

Table 2.2. Food composition of the breakfast (with milk)

Food	Serv (g)	Energy (kJ)	Energy (kcal)	Fat (g)	Sat fat (g)	Carbs (g)	Sugars (g)	Fibre (g)	Prot (g)
Wholemeal bread	64	620	147	2	0	24	3	5	7
Free range eggs	110	602	144	10	3	<0.1	<0.1	0	14
Olive light spread	15	115	28	3	1	0	0	0	0
Pineapple	125	238	56	0	<0.1	12	12	2	1
Semi skimmed milk	40	84	20	1	0	2	2	0	1
Total	357	1658	395	16	4	38	17	7	23

Serv, serving; *Sat*, saturated; *Carbs*, carbohydrates; *Prot*, protein

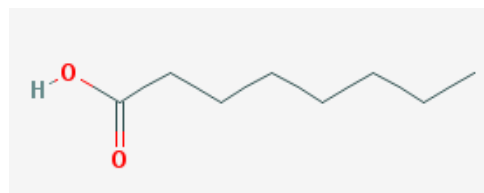
2.7.2. Breath samples collection

Breath samples were collected by blowing into a small glass tube (Labco Exetainer, Labco Limited, UK) through a straw while having the nose blocked with a nose-clip (Fig. 2.11). Participants blew into the tube while removing the straw to immediately cap the tube which was then stored at room temperature for analysis. Breath samples containing the oxidised octanoic acid (i.e. CO_2) were analysed using an isotope ratio mass spectrometer (ABCA, Sercon Ltd, Cheshire UK). The atomic mass of $^{13}\text{CO}_2$ present in the sample was quantified by the deflection that the isotope (^{13}C) labelling the ingested octanoic acid created through a magnetic field. The mass of ^{13}C was expressed in Vienna Pee Dee Belemnite (V-PDB) (an international standard for known ^{13}C composition).

**Fig. 2.11.** Breath sample collection

2.7.3. Gastric emptying measurement

As mentioned in section 2.7.1, 100 μg ^{13}C octanoic acid (octanoic acid 1- ^{13}C 99% ^{13}C , Euriso-Top, France) was pipetted into the eggs before cooking to determine GE time. Octanoic acid ($\text{C}_8\text{H}_{16}\text{O}_2$) is a medium-chain triglyceride (MCT) formed by eight carbons (Fig. 2.12) that can be naturally found in foods such as coconut oil (PubChem (n.d.)). During

**Fig 2.12.** Octanoic acid structure (PubChem (n.d.))

digestion, once MCTs reach the small intestine, they do not go into the lymphatic system as a chylomicron to get access into the venous system like long-chain triglycerides, rather they are

directly transported to the liver to be rapidly oxidised via the portal venous system (Bloom et al 1951). Thus, CO_2 appears almost immediately in the breath once the octanoic acid reaches the stomach from the duodenum. Therefore if octanoic acid is labelled with an isotope, for example ^{13}C , the GE time can be estimated by the appearance of $^{13}\text{CO}_2$ in the breath. Despite not being a direct measurement of gastric emptying, breath collection is a less invasive and expensive method than the γ scintigraphy technique, requires little training for the measurer and shows a low intra-individual variability (Perri et al 2006).

GE time can be expressed as four different parameters (Fig. 2.13):

- half time (t_{half}) which represents the time (min) that requires half of the given amount of ^{13}C to be excreted by breath;
- lag phase (t_{lag}) which represents the time (min) needed to reach the highest excretion rate of ^{13}C which coincides with the inflection point;
- latency phase (t_{lat}) which signifies the initial delay in the excretion curve and is located at the point of intersection between the tangent at the inflection point of the cumulative percentage of the dose recovered (PDR) curve and the x-axis (min);
- and ascension time (t_{asc}) which is the time period between the t_{lat} and the t_{half} representing the phase of high $^{13}\text{CO}_2$ excretion rates (min).

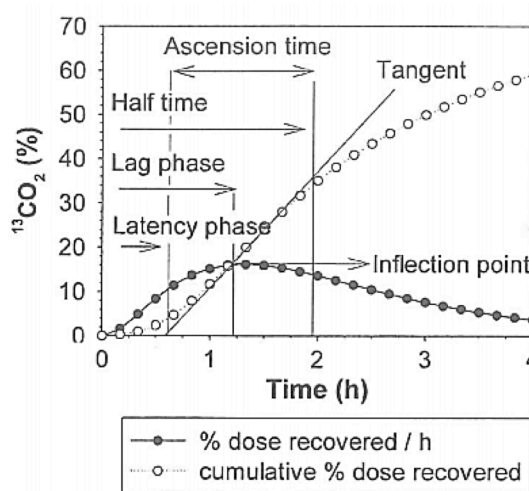


Fig 2.13. GE time points (Clegg and Shafat 2010)

To obtain these four parameters, the first step was to convert the V-PDB of $^{13}\text{CO}_2$ of each sample into moles and calculate the excess of CO_2 produced in each sample ($^{13}\text{C}_{\text{tx}}$) from the measurement taken at baseline ($^{13}\text{C}_{\text{t0}}$) when the participant was still fasted:

$$\text{Excess of } \text{CO}_2 \text{ produced (mmol)} = (^{13}\text{C}_{\text{tx}} - ^{13}\text{C}_{\text{t0}}) \times \text{CO}_2 \text{ produced}$$

where CO_2 produced equals the normal endogenous production of 300 mmol CO_2 per m^2 of body surface area (BSA) per h (Shreeve et al 1970). Participants' BSA was calculated as follows (Haycock et al 1978):

$$BSA (m^2) = weight (kg)^{0.5378} \times height (cm)^{0.3964} \times 0.024265$$

The amount of $^{13}CO_2$ produced that was formed only from the octanoic acid given was calculated:

$$Excess\ 13C_{administrated}(mmol) = (MF_{substrate} - MF_{t0}) \times \left(\frac{m}{M}\right) \times n$$

Where $MF_{substrate}$ = molar fraction of ^{13}C (0.99); m = the administered dose of ^{13}C (99mg); M = the molar mass of the ^{13}C (145mg/mmol); n = number of labelled positions in the substrate (1).

Finally, excess CO_2 produced was expressed as the PDR from that given in the breakfast:

$$PDR = \frac{excess\ CO_2\ produced}{excess\ CO_2\ administrated} \times 100$$

This was fitted into the model created by Ghooos et al. (1993) with a non-linear regression analyses using the "solver" tool of Excel (Microsoft Office, 2013):

$$y = mk\beta \times e^{-kt} \times (1 - e^{-kt})^{\beta-1}$$

Where y equals the cumulative PDR; t is the time (h); and m, k and β are constants. To ensure the validity of the model, r^2 coefficient between the raw data and the model was calculated and that equalled >0.95.

Finally, t_{half} (Ghooos et al 1993) t_{lag} , t_{asc} and t_{lat} (Schommartz et al 1997) were determined as follows: (Table 2.3):

Table 2.3. Equations used to calculate the different GE parameters (Ghooos et al 1993, Schommartz et al 1997)

GE parameter	Equation
t_{half}	$= \left[60 \left(-\frac{1}{k} \right) \times \ln \left(1 - 2^{-\frac{1}{\beta}} \right) - 66 \right] / 1.12$
t_{lag}	$= [60 \times (\ln \beta / k) - 60] / 0.94$
t_{lat}	$= \frac{1}{k} \times \left(\ln(\beta) + \frac{1}{\beta} - 1 \right)$
t_{asc}	$= -\frac{1}{k} \times \left[\ln \left(1 - 2^{-\frac{1}{\beta}} \right) + \ln(\beta) + \frac{1}{\beta} - 1 \right]$

2.8. Food intake assessment (Study 3)

2.8.1. Food diaries

During the screening session, a 3-day food diary (Appendices) along with a food weighing scale (Arc Electronic Kitchen Scale, HoMedics Group Ltd. Salter, Kent, UK) were handed out to the participants with instructions on how to complete the food diary and use the food weight scale. Food diaries are representative of a participant's food choice and eating patterns; however, errors can be made when recording meals, especially during the measurement of quantities, as participants tend to underreport (Livingstone and Black 2003). Thus, the averaged EI of the total of 9 days record (3-days x 3-MC phases) was compared to the average EE measured by the PA monitor to ensure the validity of the food diaries.

Although a 7-days food record is considered to be the gold standard of food intake assessment, it has been shown that as more consecutive days are asked to be recorded, the measurement error is magnified (Willett 2012). Therefore, a 3-day food diary (2 weekdays + 1 weekend day) is generally recommended for this practice (Rachel K Johnson 2002).

Participants were asked to weigh out and record all the foods and beverages consumed with as much detail as possible (e.g. brand, cooking process) for three days in each phase of the MC. If participants could not weigh out a meal, they were asked to provide portion sizes by using household measures (e.g. cups) and/or by taking pictures of the foods eaten. The selected days of each phase included one of the visits to the laboratory (on day 2 of the 3-days), therefore participants had to only record anything consumed after leaving the testing facilities on the test day. Before participants left the testing facilities, a new food diary was given for the next MC phase and participants were reminded to fill them in closer to the following testing day. Food intake was entered into nutrition analyses software (Nutritics V3.74 Professional Edition) and intakes of energy, carbohydrates, sugars, protein, fats, saturated fat, fibre and sodium were determined per day and per phase of the MC for each participant.

2.8.2. *Ad libitum* food intake

After the four hours of testing, a buffet lunch was provided to be consumed within 30 minutes (Fig. 2.14). This lunch was used to measure participants' food intake covertly and separately from their food diary as awareness of having their food intake assessed may change or restrain their food intake (Stubbs et al 1998). Thus, participants were told to "eat as much as you want

until you feel comfortably full". Nevertheless, it may have been obvious that food intake was measured because they did not have to recall this meal nor the breakfast of that day in their food diaries. The researcher weighed out all the foods before and after the participant had her lunch and then food intake was analysed using an excel spreadsheet designed from the manufacture's food information provided in the food label. Ad-libitum food intake assessment included the measurement of energy, carbohydrates, protein, sugars, fats, saturated fat, fibre and sodium. The foods offered in the buffet were a variety of pre-cooked dishes/foods that required little cooking for the researchers due to the lack of time of preparation during the testing. The selected foods were chosen with the aim to satisfy all tastes and possible conditions (e.g. lactose intolerance, vegetarian diets, etc.) thus food intake was not restrained by choice or quantity (see Table 2.4 for list of foods and composition). One of the participants was vegan but agreed to partake in the study because free range organic eggs were provided at breakfast. In her case the buffet was modified (five items were removed and five were exchanged) to ensure that there was enough food and variety (Table 2.4).



Fig 2.14. Buffet lunch (non-vegetarian)

Both free-living and laboratory settings present their own limitations when looking at the validity of measuring techniques for food intake. Although the latter can be very precise as the researcher is measuring the amounts of food intake in a very controlled environment, this can also have an impact on the participant's eating pattern because of being in such an artificial environment (Stubbs et al 1998). Nevertheless, this potential change produced in the eating behaviour was expected to be the similar in each session as the settings of the kitchen where participants had their lunch were kept as consistent as possible and food intake was studied by assessing within subject effects, thus minimising any bias.

Table 2.4. Foods available in buffet lunch with nutritional composition per portion provided.

Food	Serving		Energy		Fat (g)	Sat fat (g)	Carbs (g)	Sugars (g)	Fibre (g)	Prot (g)	Salt (g)
	units	g	(kJ)	(kcal)							
Hummus, T		50	664	161	14	1	4	0	2	4	1
Apples Gala, T	1	135	304	71	0	0	16	16	2	1	0
Banana, T	1	158	636	150	0	0	36	19	4	2	0
Clementines T	2	226	398	95	0	0	20	20	3	2	0
Carrots, loose, T		70	123	30	0	0	6	5	2	0	0
Celery sticks, T		80	32	8	0	0	1	1	1	0	0
Tomatoes, T	18	142	119	28	0	0	4	4	1	1	0
Potato Salad, T *		270	1858	448	35	3	28	2	3	4	1
Pasta Tuna & Sweetcorn, T		295	2295	549	25	5	59	9	3	20	1
Moroccan Couscous, T		245	2112	502	16	1	75	41	11	9	0
Bright Salad, T		83	93	22	0	0	3	3	2	1	0
Cheese, Babybel	4	95	1207	291	23	15	0	0	0	21	2
Low-fat Yoghurt, T *	1	120	406	96	1	1	19	15	0	3	0
Sausages, Lincolnshire, T	8	66	779	187	14	5	7	1	1	9	1
Chicken Nuggets, T *	5	77	770	185	10	1	14	1	1	10	0
Pizza, Cheese & Tomato, T	1	160	1934	459	12	5	68	8	2	18	1
Bread sticks, T		19	331	78	1	0	14	1	1	2	0
Crisps, ready salted, Walkers	1 bag	24	527	126	8	1	12	0	1	1	0
KitKat, Milk Chocolate	4 fingers	45	958	229	11	6	29	23	1	3	0
Orange Juice Smooth, T		500	985	230	0	0	54	41	0	5	1
Water		500	0	0	0	0	0	0	0	0	0
Egg Mayonnaise Sandwich *		139	1202	287	13	2	26	3	5	14	1
Chicken & Sweetcorn Sandwich *		139	1199	287	12	1	29	3	5	14	1
TOTAL (non-vegan buffet)		3594	17974	4291	184	42	494	192	51	142	11
Bean & Mint Salad, T Y		215	1288	310	13	2	26	2	14	15	2
Soya Fruit Yoghurt, Alpro Y	1	129	396	94	3	0	12	12	2	5	0
Vegetable Spring Rolls, T Y	6	115	1173	281	14	6	33	4	3	6	0
Peanut Butter Sandwich Y		113	1477	355	20	3	23	4	7	17	0
Beetroot & Mint hummus Sandwich Y		109	755	180	5	1	23	2	6	7	1
TOTAL (vegan buffet)		2914	11413	2721	95	16	361	175	62	78	6

Nutritional composition of the foods was based on manufacturer's information. Sat, saturated; Carbs, carbohydrates; Prot, protein, T, Tesco * Items that were removed in the vegan buffet. Y Item included in the vegan buffet only.

2.9. Appetite sensations assessment (Study 3)

Participants rated their appetite sensations before and immediately after breakfast and every 15 minutes during the first hour post-breakfast and then every 30 minutes until lunch time. Feelings of satiety were assessed by four questions (1) ‘How hungry do you feel?’, (2) ‘How full do you feel?’, (3) ‘How strong is your desire to eat?’ and (4) ‘How much food do you think you can eat?’ in which participants had to rate their appetite sensations with printed Visual Analogue Scales (VAS), namely, by putting a mark in a 100 mm line per each question, where 0 = (1) ‘not hungry at all’, (2) ‘extremely full’; (3) ‘not strong at all’ and (4) ‘nothing at all’ and 100 = (1) ‘extremely hungry’, (2) ‘not at all full’; (3) ‘extremely strong’ and (4) ‘a large amount’ (Fig. 2.15) (Appendices). The distance between the origin (score = 0) and the mark was used to measure the participant’s score.

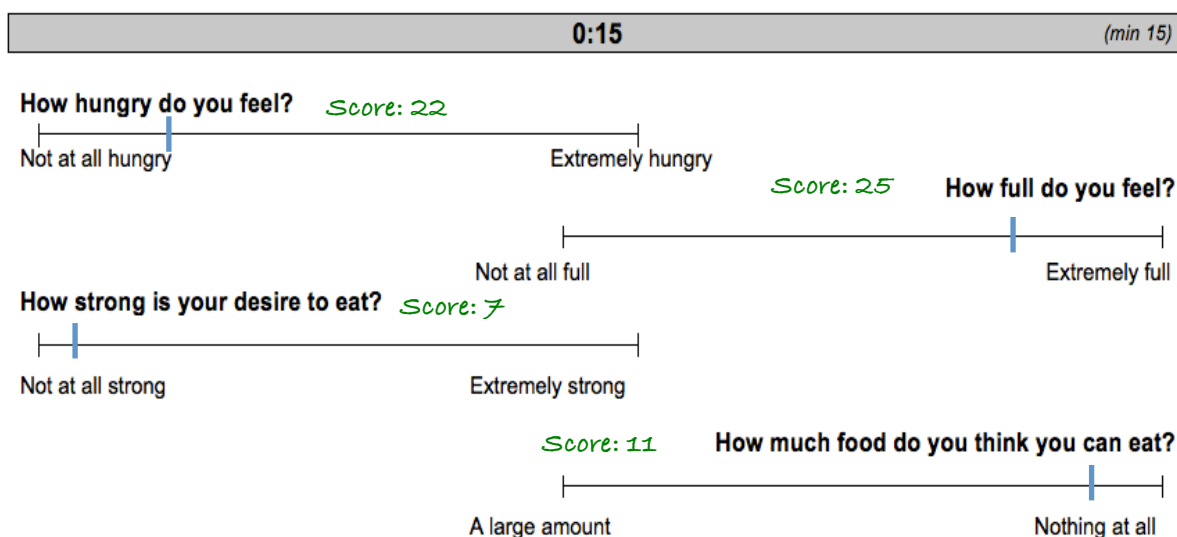


Fig 2.15. Example Visual Analogue Scale

Each question was then analysed separately by calculating the derived AUC from the scores of all the time points. AUC was calculated using the trapezoidal method at min 60, 120, 180, 210 and 240 from baseline (before breakfast). The employment of VAS has been validated in many studies and the use of total AUCs with baseline levels as covariates, has been recommended over individual time scores or incremental AUCs within participants (Blundell et al 2010).

2.10. Physical activity assessment (Study 3)

Physical activity patterns were assessed using a body monitoring system (SenseWear®, BodyMedia). The SenseWear monitor has been validated as a reliable tool to measure EE in

adults in free-living conditions (St-Onge et al 2007). The purpose of using this method instead of others, e.g. PA diary, was to obtain valid data while adding minimal disruption to the participants' normal life and to maintain recruitment and compliance in the study. The SenseWear consisted of an armband that assessed PA and lifestyle (e.g. sleeping hours) by measuring (1) motion and (2) the steps taken with a 3-axis accelerometer, and (3) the galvanic skin response (i.e. the change in the electrical conductivity of the skin due to sweat or emotional stimuli), (4) the skin temperature and (5) the heat flux with different sensors. Participants were requested to wear the activity monitor on the upper right arm (triceps muscle) throughout the day (24 hours) except during activities in which the skin is in contact with water (e.g. showering) as the equipment instructions advise (Body Media 2006). Data was downloaded and analysed as total daily energy expenditure (kcal/d) using the BodyMedia software once individual characteristics (i.e. date of birth, height, weight, sex) were entered into the system.

2.11. MC phases definition

Due to the main aim of this thesis, i.e. to determine whether changes in the ovarian hormones affects different metabolic and appetite-related factors, it was deemed appropriate to divide the MC according to the levels of the ovarian hormones. For Studies 1 and 2, measurements of E_2 and P_4 were taken three times a week during a whole cycle, thus hormone profiles could be mapped very easily and the MC in the NC group could be divided in three phases as follows: (1) MPh from day 1 until the last day of bleeding; (2) FPh from the subsequent day until the day after the E_2 peak; and (3) LPh that ended on the day prior to the start of the next cycle. This division was designed to find low concentrations of both hormones in the MPh, high E_2 levels in the FPh and high E_2 and P_4 in the LPh. Cycles that did not display a minimal concentration of 15.9 nmol/l were considered nonovulatory or not normal (Piers et al 1995). In the HC group, the MC was divided into two stages: (1) on HC stage (ON), i.e. the three weeks when the HC was used; and (2) off HC stage (OFF), i.e. the seven days when the HC was not used.

When hormones were only measured three times during the MC (Study 3), MC phases were assigned before the participant attended the laboratory and only after hormonal measurements could confirm the correct phase. Scheduling test sessions to the MPh did not present any difficulty as the participant notified the researcher of the onset of the menses and a visit to the laboratory was arranged as soon as possible; however, to determine when the

LPh began was more challenging as it is well documented that MC phase length can vary considerably amongst women and even within individuals (Fehring et al 2006). Therefore, a fertility monitor (Clear Blue Advanced Fertility Monitor) was used to measure urine levels of estrone-3-glucuronide (E3G) (the main metabolite of E_2) and LH. The advantage of these type of fertility monitors compared to others is that the LH peak, i.e. ovulation, can be anticipated by tracking E3G concentrations from day 6 of the cycle (Clearblue 2013). Moreover, it has been proved to be a valid tool when arranging laboratory visits upon the phases of the MC (Howards et al 2009).

Participants were asked to test their first urine of the morning by urinating on a stick for three seconds and then placing into the monitor to obtain a result. The monitor displayed three possible readings: 'low' (E3G levels low), 'high' (E3G levels increased) and 'peak' (LH levels high). When participants obtained the 'high' reading they notified the researcher who then scheduled the next testing session based on the day of the cycle the participant was on, her MC length history and the fact that it usually takes approximately five days to reach to 'peak' after a 'high' reading (Howards et al 2009), in order to test the participant at very high levels of E_2 . Once the 'peak' reading appeared, the last session was scheduled to test when P_4 was at its highest values (in the mid-luteal phase) based on the peak day and the usual MC length of the participant. When participants did not reach 'peak' they were asked to postpone their LPh testing until the next cycle to ensure that the P_4 levels were high enough to produce any potential effects on the parameters studied (i.e. PYY response, GE time, appetite feelings and food intake).

2.12. Statistical analyses

Sample size was initially estimated by previous similar studies and after was calculated retrospectively with the outcome data and using G*Power 3.1.9.2 (Universität Kiel, Germany) software. Data was analysed with SPSS Statistics 19. The normality of the data was tested using the Shapiro-Wilks test. When data were not normally distributed, non-parametric tests were used. Differences between groups (NC vs HC users) were compared using the independent sample t-test or Mann-Whitney U test, where appropriate. One-way repeated measures ANOVA or Friedman test was used to test differences across the phases of the MC in the NC group and paired t-test or Wilcoxon signed-rank test was used to compare between the two stages of the MC in the HC group. Statistical significance level was established at $p \leq 0.05$.

3. Ovarian hormones in plasma and saliva during the MC (Study 1)

3.1. Background

During the design of a human study, researchers should prioritise the use of testing techniques that have been shown to be reliable, affordable and, very importantly, that are less invasive for the participants taking part. Although ultrasound is the direct method to monitor the ovaries action, this may be unfeasible in many studies due to its impracticability, thus the measurement of the ovarian hormones becomes the best available option (Hampson and Young 2007). Natural MC is usually described as a “28-days” cycle in which ovulation occurs on day 14 and the FPh and LPh are equally distributed around this event. However, it is well known that MC length varies between and within individuals and that this is more dependant on the length of the FPh rather than the LPh (Fehring et al 2006). These natural variations will have an impact on the design of any MC related study as these are indicating differences in the time exposure of each of the ovarian hormones per MC.

Traditionally, when ultrasound is not available, monitoring of the MC this would be done from blood samples, but for the last decades saliva samples have progressively become a common method also employed by researchers and clinicians (Gröschl 2008). The difference between the two specimens is that blood samples are mainly composed of the bound form of the steroid hormones, whereas saliva is primarily composed of the free (bioactive) form. Moreover, it is believed that hormone concentrations obtained from saliva reflect those of unbound fraction that would be found in blood (Tenovuo 1989), thus potentially serving as a more effective biomarker than plasma levels in certain investigations where the aim is to study the influence of those on the matter investigated. Studies in the past have shown that both P_4 and E_2 levels in saliva and plasma correlate well i.e. $r = 0.68 - 0.974$ (De Boever et al 1986, Bolaji 1994, Bourque et al 1986, Walker et al 1979, Worthman et al 1990) in NC women. Nevertheless, how these two measurements relate is still not completely elucidated as saliva concentrations are not only dependant on plasma concentrations, but also on the levels of their plasma carriers (Elliott et al 2003).

To our knowledge no previous studies have looked at the correlations between plasma and salivary ovarian hormones throughout a MC in HC users. The aim of this study was to explore the patterns that the ovarian hormones display in plasma and saliva throughout the MC in a group of NC women and HC users. In particular, we aimed to study whether the levels of ovarian hormones changed significantly in the different phases of the MC in both specimens

(saliva and plasma) and how this compared between groups as an average of the MC. Furthermore, a secondary objective of this investigation was to study the correlations between saliva and plasma for E_2 and P_4 overall, per group and per phase of the MC.

3.2. Methods

3.2.1. Protocol

Once the participants were accepted to partake in the study, they were asked to notify the researcher upon the start of their menstruation (defined as day 1). Then they attended the laboratory for the measurement of the ovarian hormones in plasma and saliva on the following morning and three times a week during a whole MC, i.e. until the start of the menstruation of the next cycle (day 1 of cycle 2) (approximately 12 visits in total). The MC in the NC group was divided in three phases as follow: (1) MPh from day 1 until the last day of bleeding; (2) FPh from the subsequent day until the day after the E_2 peak; and (3) LPh that ended on the day prior to the start of cycle 2. In the HC group, the MC was divided in two stages: (1) on (ON), i.e. the 21 days when the HC was used; and (2) off (OFF) stage, i.e. the seven days when the HC was not used.

On the testing day participants attended the laboratory in the morning (between 07:00-10:00) after a 10-12 hour overnight fast. After an hour in the laboratory, they rinsed their mouth and after 5 min a ~2 ml saliva sample was collected via passive drool and under standardised conditions (see chapter 2.5.1). Saliva samples were kept in ice until they were distributed in different aliquots to be stored at -40°C within four hours of collection until they were analysed to measure their contents of free E_2 and P_4 by ELISA. Variability between salivary E_2 and P_4 assay duplicates was 5.8 ± 6.5 and 4.0 ± 4.3 (%CV), respectively. Following that, a venous blood sample of 6 ml was collected for the measurement of total E_2 and P_4 . Blood samples were centrifuged to extract their plasma; this was then stored at -80°C for future analyses by ECLIA.

3.2.2. Calculations and statistical analyses

Sample size was based on study 3 calculation (see chapter 4 for more details).

Data were analysed using SPSS Statistics 19. Values are expressed as means \pm SD for descriptive parameters and means \pm SE for measurements expressed as averages of the

different phases. The normality of the data was tested using the Shapiro-Wilks test. When data were not normally distributed, non-parametric tests were used. Differences in ovarian hormones between groups were compared using the independent sample t-test or Mann-Whitney U test, where appropriate. One-way repeated measures ANOVA or Friedman test was used to test differences in averaged hormones levels in plasma and saliva and the ratio between the two specimens across the phases of the MC in the NC group and paired t-test or Wilcoxon signed-rank test was used when comparing the two stages of the MC in the HC group. Analyses were also performed without averaging concentrations of hormones per participant and phase, therefore all the data points were used to compare between phases and groups. Correlations between specimens were analysed by Pearson's or Spearman's correlation using each data point for the whole sample of visits as well as per each group and phases of the MC.

Statistical significance level was established at $p \leq 0.05$ except in the Wilcoxon tests (used for pairwise comparisons following the Friedman tests) when a Bonferroni correction was applied so that all effects are reported at a 0.0167 level of significance.

3.3. Results

3.3.1. Participants characteristics

Of the 27 women (16 NC and 11 HC users) who started the study, two withdrew due to personal reasons, two were excluded because they did not fulfil the inclusion criteria, e.g. cycle length <25 days PCOS, and another due to an incomplete data set (see chapter 2.2). Most HC participants were using a combined HC, thus one participant who was using a P₄-only HC was

Table 3.1. Formulation of the different hormonal contraceptives used by participants.

<i>n</i>	Trade name (form)	Hormone Dose		
		EthinylEstradiol	Progesten	
1	Leeloo Ge (oral)	20 µg	100 µg	levonorgestrel
1	Loestrin 20 (oral)	20 µg	1000 µg	norethisterone
2	Gedarel (oral) / Marvelon (oral)	30 µg	150 µg	desogestrel
1	Microgynom (oral)	30 µg	150 µg	levonorgestrel
1	Evra (patch)	34 µg	203 µg	norelgestromin
1	Cilest (oral)	35 µg	250 µg	norethisterone
2	Dianette (oral) / Midane (oral)	35 µg	2000 µg	cyproterone acetate

also excluded from the study as her ovarian hormones pattern was very different to what was seen in the NC or HC women. The HC formulations can be found in Table 3.1.

Therefore, the results presented are based on a sample of 20 healthy women (11 NC and 9 HC users). The majority of the women were Caucasian (6 NC and 9 HC users) and the rest had an Arab (4 NC) or Asian (1 NC) ethnic background. Table 3.2 presents the physical characteristics of the participants. There were no significant differences in any of these variables between the two groups except for waist circumference ($p = 0.041$).

Regarding the MC characteristics, the average length for the whole MC, the MPh, FPh and LPh was 29 ± 3 , 5 ± 1 , 13 ± 5 and 11 ± 3 days, respectively in the NC group. In the HC group, 28 ± 1 , 21 ± 1 and 7 ± 1 days was the averaged count for the MC, ON and OFF stages, respectively.

Table 3.2. Baseline characteristics of the NC and HC groups.

	NC (n 11)	HC (n 9)	p
Age (y)	27 ± 6	23 ± 4	0.089
BW (kg)	65.5 ± 11.6	57.5 ± 9.2	0.131
Height (m)	1.67 ± 0.08	1.66 ± 0.06	0.710
BMI (kg/m^2)	23 ± 3	21 ± 2	0.063
FM (kg)	20.2 ± 7.2	15.7 ± 7.3	0.213
FM (%)	29.8 ± 6.1	26.2 ± 8.0	0.305
FFM (kg)	45.4 ± 5.1	41.8 ± 3.6	0.107
Waist (cm)	73.3 ± 6.5	67.5 ± 4.3 *	0.041
Hip (cm)	101.1 ± 7.6	95.8 ± 8.0	0.167
Waist-to-hip ratio	0.73 ± 0.03	0.71 ± 0.05	0.307

Means \pm SD. BW, body weight; FM, fat mass; FFM, fat free mass.

* Significantly different to NC.

3.3.2. Changes in ovarian hormones across the phases of the MC

As an average of the MC, E_2 and P_4 levels in plasma were significantly higher in the NC than the HC group (Table 3.3). In saliva, only E_2 levels were significantly higher in the NC compared to the HC women, whereas P_4 levels were kept at similar levels (Table 3.3).

Table 3.3. Ovarian hormone levels in plasma and saliva as an average of the MC in the NC and the HC group.

	NC (n 11)	HC (n 9)	p
Plasma E_2 (pg/ml)	147.3 ± 8.1	21.1 ± 3.6 *	<0.001
Plasma P_4 (ng/ml)	5.20 ± 0.52	0.57 ± 0.09 *	<0.001
Salivary E_2 (pg/ml)	2.58 ± 0.14	2.03 ± 0.24 *	0.049
Salivary P_4 (pg/ml)	122.8 ± 20.3	125.6 ± 28.2	0.937

Means \pm SE. * Significantly different to NC.

In the NC group, significant changes were observed throughout the phases of the MC in plasma E_2 $\chi^2(2, N= 11) = 16.55$, and P_4 $\chi^2(2, N= 11) = 15.27$ ($p < 0.001$ in both). In particular, E_2 was significantly higher in the FPh and LPh (median = 87.7 and 167.5 pg/ml, respectively) compared to the MPh (median = 41.3 pg/ml), $z = -2.93$, $p = 0.003$ for both, whereas P_4 was significantly increased in the LPh (median = 10.69 ng/ml) compared to the MPh (median = 0.97 ng/ml), $z = -2.85$, $p = 0.004$ and the FPh (median = 0.80 ng/ml), $z = -2.93$, $p = 0.003$ (Fig. 3.1).

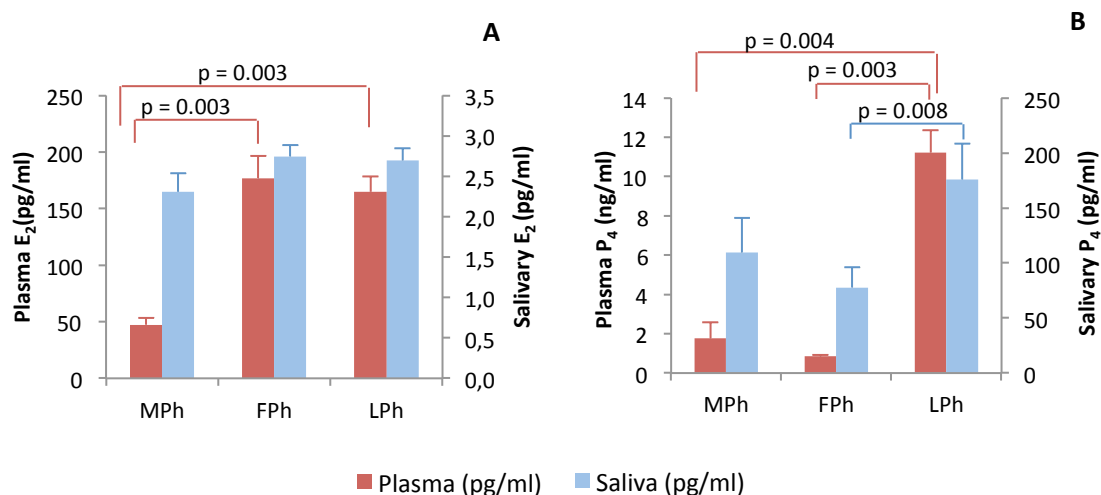


Fig. 3.1. E_2 (A) and P_4 (B) levels in plasma and saliva in the different phases of the MC in the NC group (means \pm SE)

Although the ovarian hormones in saliva showed a similar pattern as in plasma (see Fig 3.2), changes in salivary E_2 did not reach statistical significance across the phases of the MC ($p = 0.059$) (Fig 3.1). Nevertheless, salivary P_4 changed significantly throughout the MC $\chi^2(2, N= 9) = 10.89$, $p = 0.004$, showing significantly higher levels in the LPh (median = 162.6 pg/ml) compared to the FPh (median = 61.2 pg/ml), $z = -2.67$, $p = 0.008$, but not the MPh (median = 72.2 pg/ml), $z = -1.60$, $p = 0.110$ (Fig 3.1).

When looking at the ovarian hormones in the HC users, a different fluctuation pattern to the NC women was observed throughout the MC. As depicted in Fig. 3.2, while plasma P_4 was constantly kept at very low levels (similar to that seen in plasma concentrations during the MPh and FPh of the MC), plasma E_2 experienced a peak during the weeks OFF the pill just before participants started using the HC again. Thus, plasma E_2 was significantly higher in the weeks OFF the pill by almost two fold ($Z = -2.073$, $p = 0.038$) (Fig 3.3).

In saliva, P_4 levels were constantly kept at similar levels in the different weeks of the MC in the HC group ($p = 0.483$) (Fig 3.3), although individual concentrations ranged widely with some showing similar levels to those observed in the NC group (19.28 - 259.99 pg/ml). Similarly, salivary E_2 levels were marginally changed ($p = 0.144$).

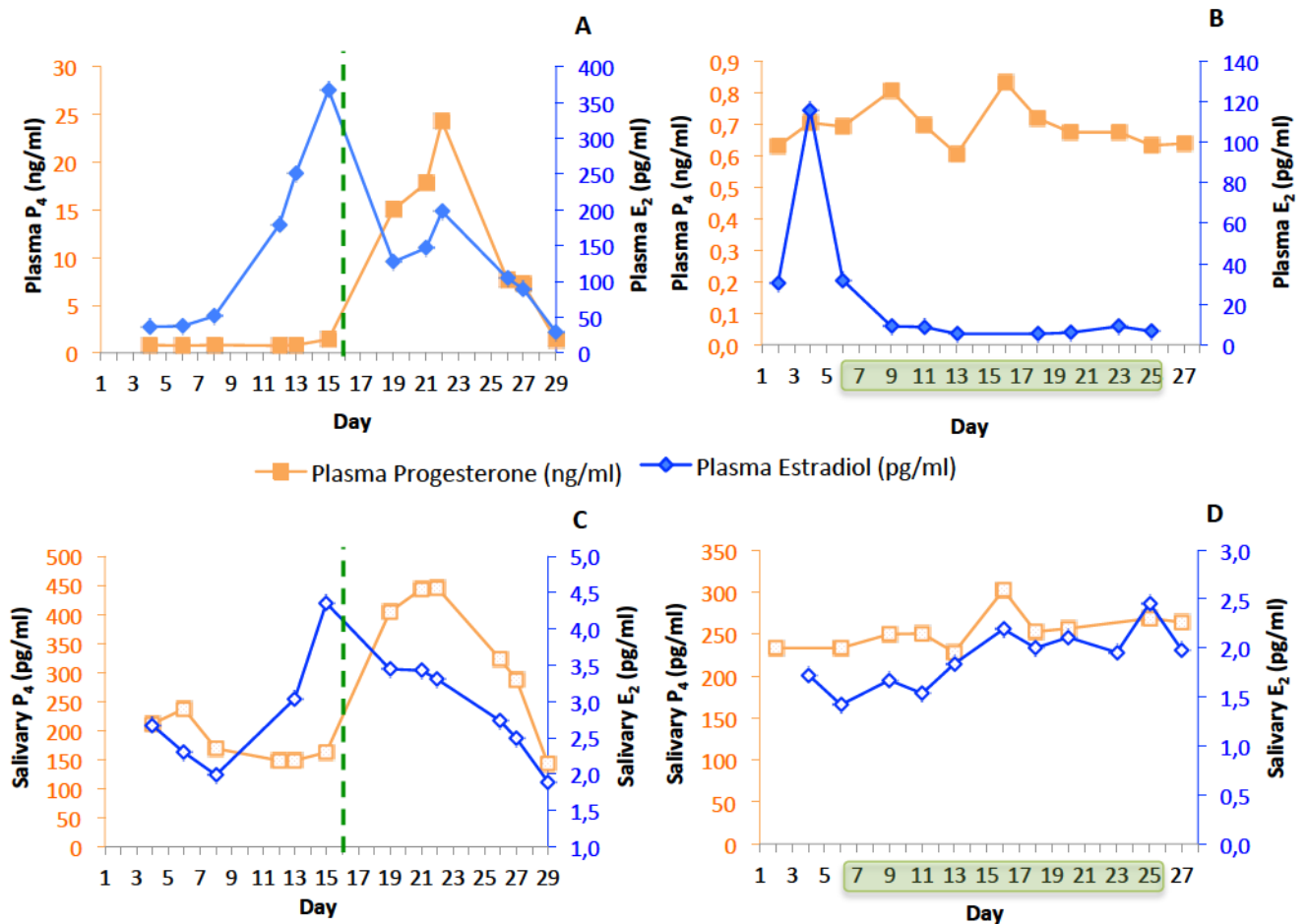


Fig 3.2. Examples of plasma (A, B) and salivary (C, D) ovarian hormones throughout the MC in a NC participant (right) and a HC participant (left). Dashed line indicates ovulation in NC woman; highlighted days correspond to the ON stage in HC user.

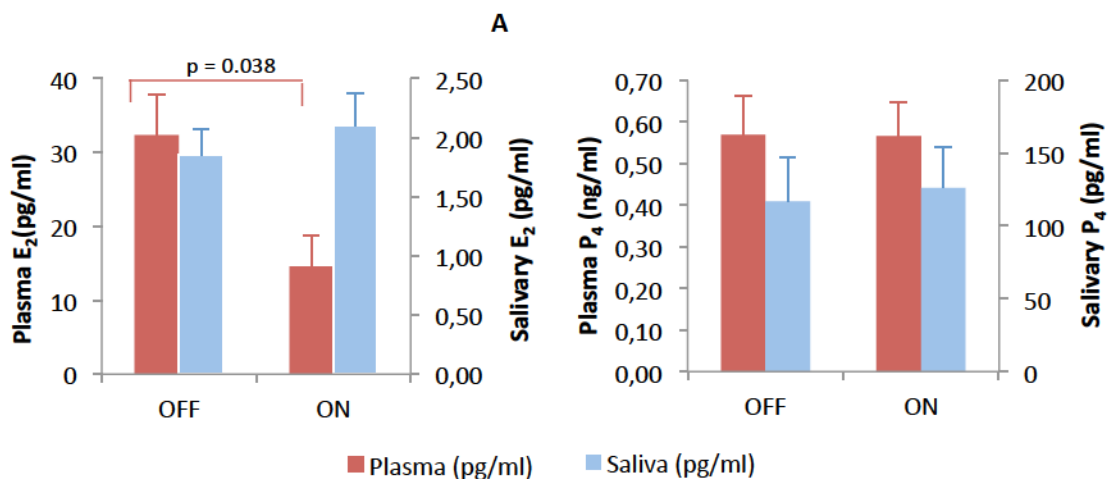


Fig. 3.3. E_2 (A) and P_4 (B) levels in plasma and saliva in the different phases of the MC in the HC group (means \pm SE)

3.3.3. Ratios and correlations between specimens

Saliva:plasma ratios in the specimens changed for E_2 ($p < 0.001$) and P_4 ($\chi^2(2, N = 9) = 10.89$, $p = 0.004$) across the phases of the MC in the NC (Fig 3.4). Ratio of specimens for E_2 was significantly higher in the MPPh compared to the other phases (Fig 3.4) and ratio of specimens for P_4 was significantly lower in the LPh compared to the other phases. In the HC group, ratio between specimens changed significantly for E_2 ($p = 0.026$), being ~ 60% greater in the weeks ON than in the OFF, but not for P_4 (Fig 3.4).

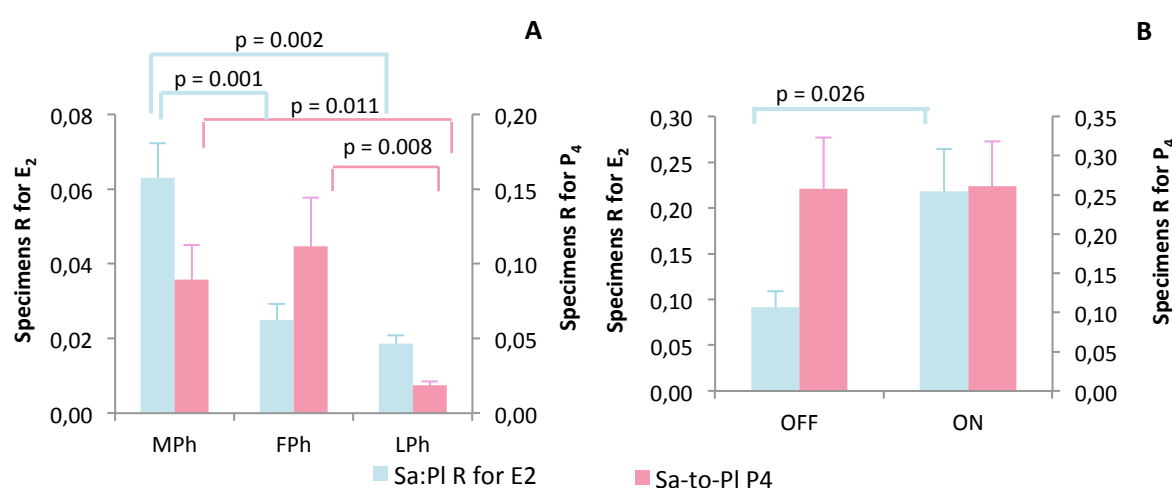
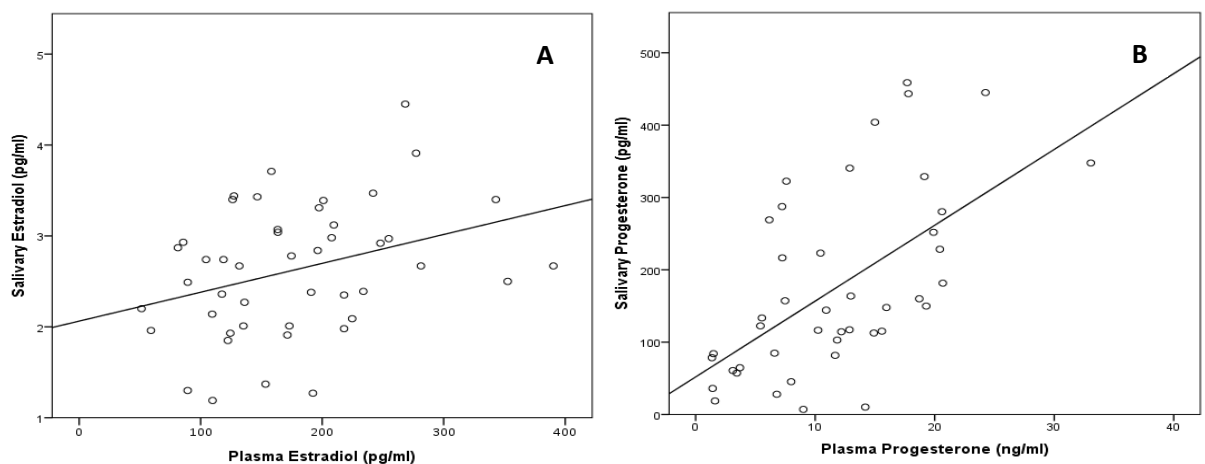


Fig 3.4. Saliva:plasma ratio for E_2 and P_4 in the different phases of the MC in the NC (A) and the HC (B) groups (means \pm SE)

Regarding, the correlations between the two specimens measuring the ovarian hormones, the results showed a weak, but significant, correlation between salivary and plasma E_2 ($r = 0.290$, $p < 0.001$) but not for P_4 ($r = 0.111$, $p = 0.145$) when looking at all the measurements with the two groups combined ($n = 160$ and 175 for E_2 and P_4 , respectively). However, when selecting the measurements of only the NC women ($n = 111$ and 99), significant weak correlations were found for both hormones, ($r = 0.227$; $p = 0.017$ and $r = 0.352$; $p < 0.001$ for E_2 and P_4 , respectively). Nevertheless, further analyses per each phase of the MC (Table 3.4) showed that solely in the LPh significant correlations existed between the two specimens and that only P_4 was strongly correlated ($r = 0.607$, $p < 0.001$) (Fig 3.5).

Table 3.4. Correlations between saliva and plasma for E₂ and P₄ levels in the different phases of the MC in the NC group.

		MPh	FPh	LPh
E ₂	<i>r</i>	-0.264	0.225	0.343
	<i>p</i>	0.236	0.113	0.020
	<i>n</i>	22	51	46
P ₄	<i>r</i>	0.135	0.249	0.607
	<i>p</i>	0.559	0.111	<0.001
	<i>n</i>	21	42	43

Bold indicates significant correlation**Fig 3.5.** Correlations between plasma and salivary E₂ (A) and P₄ (B) concentrations in the LPh

In the HC group, there were no correlations between saliva and plasma for E₂ and P₄ when looking at the whole cycle or in each phase (Table 3.5).

Table 3.5. Correlations between saliva and plasma for E₂ and P₄ levels in the HCs group and in their different stages of the MC in the HC group.

	MC	ON	OFF
E ₂	-0.028	0.156	-0.108
	0.847	0.371	0.714
	49	35	14
P ₄	0.159	0.205	0.084
	0.170	0.130	0.724
	76	56	20

Bold indicates significant correlation

3.3.4. Ovarian hormones analysed as averages of all the data points

This section aimed to study the ovarian hormones between groups and within the MC by analysing the data of all the visits of the participants.

Both plasma E₂ and P₄ were again significantly higher in the NC women as well as salivary E₂, but there were no differences in salivary P₄ (Table).

Table 3.6. Ovarian hormone levels in plasma and saliva as an average of all data points NC and the HC group of the MC.

	NC	HC	<i>p</i>
Plasma E ₂ (pg/ml)	146.0 ± 118.7	24.4 ± 26.0 *	<0.001
Plasma P ₄ (ng/ml)	5.13 ± 7.28	0.55 ± 0.23 *	<0.001
Salivary E ₂ (pg/ml)	2.20 ± 0.67	2.67 ± 0.70 *	<0.001
Salivary P ₄ (pg/ml)	132.85 ± 104.11	137.44 ± 83.63	0.383

Means ± SD * Significantly different to NC.

Within the MC of the NC women, plasma E₂ and P₄ changed significantly ($p < 0.001$ for both). E₂ was significantly lower in the MPh compared to the FPh and LPh (49.8 ± 29.5 vs. 163.1 ± 149.0 and 172.9 ± 77.8 pg/ml, respectively) ($p < 0.001$ for both), and also was significantly higher in the LPh compared to the FPh ($p = 0.014$). P₄ was significantly higher in the LPh than the MPh and FPh (11.49 ± 7.72 vs. 2.04 ± 5.32 and 0.84 ± 0.35 ng/ml, respectively) ($p < 0.001$ for both).

The salivary hormones displayed the same pattern, but once again only P₄ levels fluctuated significantly within a MC, being significantly higher in the LPh compared to the MPh and FPh (174.6 ± 124.7 vs. 99.4 ± 82.1 and 100.2 ± 63.6 pg/ml, respectively) ($p = 0.014$ and 0.010 , respectively). Salivary E₂ levels were 2.42 ± 0.76 , 2.82 ± 0.64 and 2.63 ± 0.71 for the MPh, FPh and LPh, respectively ($p = 0.166$). The ratio between specimens was affected by the phase of the MC for both, E₂ and P₄ ($p < 0.001$) (table).

Table 3.7. Saliva:plasma ratio for E₂ and P₄ in the different phases of the MC in NC from all data points.

	MPh	FPh	LPh	<i>p</i>
E ₂ ratio	0.06 ± 0.04^a	0.03 ± 0.02^b	0.002 ± 0.01^c	<0.001
P ₄ ratio	0.10 ± 0.08	0.15 ± 0.11	0.02 ± 0.01^a	<0.001

Means ± SD. Different superscripts denote significant differences between phases.

In the HC group, only plasma E₂ was significantly increased in the OFF stage compared to the ON (34.9 ± 27.2 and 19.7 ± 24.4 pg/ml, respectively). The ratio between specimens only

changed significantly in E_2 , being significantly lower in the OFF stage compared to the ON stage (0.10 ± 0.07 vs. 0.20 ± 0.12 , respectively).

3.4. Discussion

This study set out with the aim of exploring the patterns that the ovarian hormones exhibit throughout a MC in plasma and saliva in a group of NC women and HC users. Our findings are in agreement with the literature which states that plasma E_2 levels are increased in the FPh and LPh of the MC while P_4 is raised in the LPh of the MC in NC women (Hampson and Young 2007). Nevertheless, E_2 levels in plasma were also significantly higher in the LPh than the FPh when analysing the data per individual points, rather than when comparing averages of phases within participants. This is somehow surprising but it might have been affected by the fact that the FPh can include days in which E_2 levels are still very low, the days immediately after the cease of the MPh and therefore, lowering the average E_2 concentrations of the FPh.

Although P_4 in saliva fluctuated similarly to that in plasma, only when analysing all data points, salivary P_4 levels were significantly higher in the LPh than the MPh. A possible reason for not reaching the statistical difference between these two phases when analysing the average concentrations might be due to the between individual differences.

In contrast, significant changes within a MC were not found in salivary E_2 despite showing a strong trend ($p = 0.057$) towards the pattern seen in plasma. This was consistent when all data points were used for the analyses. A reason behind these dissimilarities between specimens could rely on not having a big enough sample or rather, because salivary E_2 does not change as robustly as in plasma (18 vs. 73% difference in concentrations between the MPh and FPh in saliva and plasma, respectively). In fact, this was seen by Elliott et al (2003) who measured E_2 one day in the MPh and another in the mid-LPh (after confirmed ovulation) and found no differences in salivary levels but found significant differences in plasma. Our results suggest that although (total) E_2 in plasma is significantly lower in the MPh compared to the other phases, its expected unbound levels are kept constant throughout the phases of the MC, thus having an impact on the significantly higher saliva:plasma ratio in the MPh compared to the other phases.

With the purpose of understanding the mechanisms underlying these changes in free E_2 in plasma, special attention needs to be given to the SHBG as this is the main plasma carrier

(shared with testosterone), therefore its concentration becomes crucial (Tenovuo 1989). Different studies have shown how SHBG concentrations vary across the phases of the MC, being significantly higher in the FPh and LPh compared to the MPh (Day et al 2005, Rothman et al 2011). Assuming this also occurred in our participants, it could be argued that SHBG augmented more robustly than total E_2 , thus reducing the fraction of free E_2 . This has been seen in studies looking at total and free testosterone levels across the MC (Rothman et al 2011).

Likewise, P_4 saliva:plasma ratio changed significantly, being lower in the LPh compared to the other phases as a result of an inflated increase from the FPh to the LPh in plasma P_4 compared to the rise observed in saliva (~13 vs. ~2 fold increase, respectively). Similar changes in the ratio between specimens have been described by others i.e. from 0.053-0.096 in the FPh to 0.011-0.016 in the LPh (Bourque et al 1986, Cedard et al 1984).

Interestingly, changes in the ratio between specimens throughout the MC were consistent despite the analyses performed, i.e. from the average of each phase within participants or by pooling all data together per MC phase. Alterations in the ratio between specimens across the MC inevitably had an impact on the correlations. The findings of the present study revealed that E_2 and P_4 levels measured in saliva and plasma were significantly correlated but with weak association in the NC women. The weak correlation in P_4 was somehow unexpected as others have found stronger associations i.e. $r = 0.68 - 0.974$) (De Boever et al 1986, Bolaji 1994, Bourque et al 1986, Walker et al 1979). Nevertheless, a recent study that employed not only the same technique (ELISA) but also the same reagent kit (Salimetrics) for the measurement of salivary P_4 , found that correlation levels between saliva and dried blood spot samples (a previously tested method) were similar to those seen in our study ($r = 0.345$) (Konishi et al 2012). Thus, it might be that the technique employed in the present study was not sensitive enough to accurately determine salivary P_4 . However, our results showed an improved correlation when only selecting the days within the LPh ($r = 0.607$), which was supported by the changes in the ratio between saliva and plasma throughout the MC. This agrees with the findings of Bourque et al (1986) in which saliva and plasma P_4 correlation was improved from $r = 0.68$ to $r = 0.78$ by calculating the associations only in LPh. Furthermore, their results also showed a non-existent correlation during the FPh. Finally, they demonstrated that levels of salivary P_4 were unaffected by changes in the saliva flow rate.

The weaker correlation seen in the ratio between specimens for E_2 in the LPh ($r = 0.343$) could be related to inter-individual differences between women. This has been previously suggested

by Lu et al (1999) where not only women presented a wide range of individual correlations for E_2 collected in serum and saliva ($r = 0.40 - 0.85$), but also one of the participants had a negative correlation which may have affected the opportunity of finding a significant correlation between specimens as a group. Nevertheless, it is worth noting that although blood samples were collected in the morning, saliva samples were collected in the evening, which may have had an important effect on the levels of E_2 as shown by the fluctuations that occur within a day (Bao et al 2003).

As expected, E_2 and P_4 levels in plasma were significantly decreased in the HC group by the action of the drug impairing the synthesis of the endogenous hormones as the two gonadotropins, FSH and LH, are inhibited (Monga and Dobbs 2011). Nevertheless, salivary concentrations were only significantly different between groups for E_2 . The analyses method had no impact on the results when comparing the two groups. This was surprising although Liening et al (2010) encountered similar results in a less controlled study. Furthermore, this becomes more unforeseen when levels of CBG, i.e. the main P_4 transporter, would have been expected to increase due to the use of HC (Endrikat et al 2002), thus, in theory, impairing the availability of free P_4 . A possible reason for this might be related to the measurement assays employed; there might have been a cross-reaction with the HC progestins that resulted in a false positive in the assessment of endogenous P_4 levels.

Regarding the changes within the cycle of the HC users, neither salivary E_2 nor P_4 varied significantly between the two stages. Nonetheless, saliva:plasma ratio in E_2 was decreased in the OFF phase due to the significant increase in E_2 in plasma (but not in saliva) at that phase. SHBG has been shown to increase ~1.6-fold during OFF compared to ON stage (Endrikat et al 2002), which might explain the insufficient increase in free (salivary) E_2 during OFF, thus affecting the ratio in specimens between stages. The analyses method had no impact on the results when comparing the two phases of the HC users MC.

To our knowledge, no studies have looked at the associations between saliva and plasma in the ovarian hormones in HC users. The present study showed no correlations between specimens in this group. Reasons behind this discrepancy between groups might relate to the known changes in the regulation and the concentrations of SHBG and testosterone induced by the HC (Zimmerman et al 2014). If concentrations of salivary P_4 corresponded to the progestins of the HC rather than the endogenous hormone, our results for this parameter would be invalid in this group. Further research is required to assess the salivary concentrations of P_4 in HC users with an ELISA assay as results from a study using a radio-immunoassay have indeed

shown how HC users present significantly lower levels in salivary P_4 than in NC women (Schultheiss et al 2003).

Although the purpose of our study was exploratory rather than mechanistic, the interpretation of our findings was limited by the lack of some measurements such as SHBG, testosterone and CBG concentrations as these are determinants of total and free E_2 and P_4 concentrations.

To conclude, our findings showed that salivary ovarian hormones, which represent the unbound fraction of the concentrations in plasma, are not in a linear relationship with those in plasma across the MC because of the changing ratio between the two specimens throughout the phases of the MC. In addition, fluctuations in saliva are in a much smaller proportion compared to those observed in plasma which may be affected by changes in the SHBG. Finally, HC users showed suppressed plasma ovarian hormones as a result of the use of the HC. This was also seen in salivary E_2 , but could not be seen in P_4 due to cross-reactivity with the progestins of the HC.

4. RMR and substrate metabolism during the MC (Study 2)

4.1. Background

Daily total energy expenditure encompasses BMR, PA and dietary-induced thermogenesis. From these three constituents, the most important is BMR or RMR as it can account for up to 70% of the total energy expenditure (Payne and Waterlow 1971). Hence, the determination of BMR/RMR is crucial for estimating energy requirements, particularly in overweight and obese populations which are more likely to obtain inaccurate estimations from the predictive equations (i.e. >10% difference to the actual RMR) (Frankenfield et al 2003).

Age, body composition, sex, race and physical fitness are features that have been shown to influence RMR (Arciero et al 1993, Forman et al 1998, Van Pelt et al 1997, Ravussin et al 1982). Furthermore, ovarian hormones might affect the daily variability of RMR as previous studies reported a higher (intra-) coefficient of variation (CV%) in women than men, i.e. 1.7-10.4 vs. 2.5-4.0 % (Diffey et al 1997, Henry et al 1989, 2003, Soares and Shetty 1987). In fact previous research has shown that women might experience a cyclical pattern in energy demands characterised by an increase in RMR after ovulation until the start of menstruation (Solomon et al 1982, Webb 1986). Whether the increase in RMR comes from a higher expend of both energy suppliers (i.e. carbohydrates and fat) or whether it comes from an increase in mainly one of them is still uncertain although the literature suggests towards a higher fat oxidation post-ovulation (Matsuo et al 1998, 1999).

In contrast, it appears that women who are using hormonal contraceptives (HC) experience a more constant RMR throughout their cycles (Curtis, Henry and Ghossein-Choueiri 1996). This seems to support the hypothesis that ovarian hormones, or fluctuations in ovarian hormones, can induce changes in RMR as women who take HC present more stable ovarian hormone levels. Nevertheless, current hormonal contraceptive preparations are present in very different forms and concentrations, therefore how these might modulate RMR is uncertain.

Despite the necessity of measuring RMR in order to accurately determine individuals' energy requirements, under certain circumstances, due to the cost or the availability of the instruments, researchers and nutritionists rely on predictive equations of RMR. From the original Harris and Benedict equations in 1919 until the Henry equations in 2005, many equations have been formulated based on different populations (EFSA 2013). Although it is well recognised that the best predictive equations are those calculated from the population studied, it was recently accepted that the Harris-Benedict (1917), Schofield et al (1985), Mifflin

et al (1990), Müller et al (2004) and Henry (2005) equations are all equally valid for adults (EFSA 2013). Nevertheless, studies have found a lack of agreement between measured (RMR_m) and predicted RMR (RMR_p) (Weijts and Vansant 2010). Moreover, the effect of the MC on the accuracy of these predictions has never been studied before.

To our knowledge, no previous studies investigating RMR during the MC have averaged more than two days in each MC phase using blood hormone measurements to define MC phases, therefore the aim of our study was:

- To investigate whether RMR in the LPh is significantly higher than in the FPh and/or the MPh and whether this was associated with changes in the levels of the ovarian hormones.
- To study whether substrate oxidation rates and contribution to energy expenditure experienced any significant changes during the MC.
- To compare the variability of RMR and substrate metabolism between women who are taking or not HC.
- To explore how the accuracy of RMR predicted by validated equations was affected throughout a MC.

4.2. Methods

4.2.1. Protocol

Once the participants were accepted to partake in the study, they were asked to notify the researcher upon the start of their menstruation (defined as day 1). Then they attended the laboratory for the measurement of RMR, substrate oxidation, ovarian hormones and body composition on the following day and three times a week during a whole MC, i.e. until the start of the menstruation in the following cycle (day 1 of cycle 2) (approximately 12 visits in total). The MC in the NC group was divided in three phases as follow: (1) MPh from day 1 until the last day of bleeding; (2) FPh from the subsequent day until the day after the E₂ peak; and (3) LPh that ended on the day prior to the start of the next cycle. In the HC group, the MC was divided in two stages: (1) on HC stage (ON), i.e. the three weeks when the HC was used; and (2) off HC stage (OFF), i.e. the seven days when the HC was not used.

On the testing day participants attended the laboratory in the morning (between 07:00-10:00) after a 10-12 hour overnight fast and rested while lying down for 30 minutes to achieve a state

of stabilization after their commute to the laboratory. RMR and substrate oxidation were measured with a hooded open circuit indirect calorimeter GEM (GEMNutrition Ltd, Daresbury, UK) for 30 minutes under standardized conditions (Compher et al 2006) (see section 2.6). The first five minutes of measurement were automatically discarded so that the participants had enough time to adapt to the hood. Thus, the average of the remaining 25 minutes measurement was used to calculate the RMR and substrate oxidation. The room temperature where the indirect calorimeter was kept was $21.2 \pm 1.2^{\circ}\text{C}$ and a blanket was offered in case the participant felt cold. Following that, a ~2 ml saliva sample was collected via unstimulated passive drool technique and under standardised conditions (see section 2.5) to measure concentrations of E_2 and P_4 in saliva. Saliva samples were kept in ice and then stored at -40°C within four hours of collection until they were analysed with an ELISA. Finally, a venous blood sample of 6 ml was collected for the measurement of E_2 and P_4 . Samples were centrifuged to extract their plasma; this was then stored at -80°C for future analyses by ECLIA.

4.2.2. Calculations and statistical analyses

RMR was calculated as absolute RMR (i.e. per day) and relative RMR (i.e. per kg of BW per day) as RMR can be influenced by body size. Bias was determined as the mean percentage difference between RMR_m and RMR_p and accuracy as the percentage of participants with an RMR_p within $\pm 10\%$ of RMR_m.

Based on previous studies investigating differences in RMR between phases of the MC, it was determined that a sample size of 14 would be adequate to detect changes in RMR during the MC in NC women (Day et al 2005). Retrospective analyses, using the G*Power software with a partial eta square (η^2) of 0.207 and $\alpha = 0.05$, determined that there would have been an 80.0 % chance of correctly rejecting the null hypothesis of no differences in the RMR across the phases of the MC with a total of 21 participants.

Data was analysed with SPSS Statistics 19. Values are expressed as means \pm SD for descriptive parameters and means \pm SE for averaged measurements within the MC phases. The normality of the data was tested using the Shapiro-Wilks test. When data were not normally distributed, non-parametric tests were used. Levels of inter- and intra-individual variation of RMR were calculated by determining the coefficient of variation (CV %). Differences in RMR and ovarian hormones between groups were compared using the independent sample t-test or Mann-Whitney U test, where appropriate. One-way repeated measures ANOVA or Friedman test was

used to test differences across the phases of the MC in the NC group and paired t-test or Wilcoxon signed-rank test was used to compare between the two stages of the MC in the HC group. Associations between RMR and hormone levels were analysed by Pearson's or Spearman's correlation. A multiple regression model with body composition and the ovarian hormone levels (from plasma and saliva) as predictive variables was performed to predict RMR. The ratio between the two specimens (i.e. plasma and saliva) of E_2 and P_4 as well as the ratio between the two ovarian hormones for each specimen were calculated for each data point to be included in the multiple regression models. Statistical significance level was set at $p \leq 0.05$.

4.3. Results

4.3.1. Participants characteristics

Of the 26 women (15 NC and 11 HC users) who started the study, two withdrew due to personal reasons, two were excluded because they did not fulfil the inclusion criteria, i.e. cycle length <25 days, and one was excluded due to incomplete data set (see chapter 2.2). Most HC participants were using a combined HC, thus one participant who was using a P_4 -only HC was also excluded from the study as the effect of this type of HC on the production of endogenous hormones was different to that observed in the combined HC users. The HC formulations can be found in table 4.1.

Table 4.1. Formulation of the different hormonal contraceptives used by participants.

<i>n</i>	Trade name (form)	Hormone Dose		
		Ethinylstradiol	Progesten	
1	Leeloo Ge (oral)	20 µg	100 µg	levonorgestrel
1	Mercilon (oral)	20 µg	150 µg	desogestinel
1	Loestrin 20 (oral)	20 µg	1000 µg	norethisterone
2	Gedarel (oral) / Marvelon (oral)	30 µg	150 µg	desogestrel
1	Microgynom (oral)	30 µg	150 µg	levonorgestrel
1	Evra (patch)	34 µg	203 µg	norelgestromin
1	Cilest (oral)	35 µg	250 µg	norethisterone
2	Dianette (oral) / Midane (oral)	35 µg	2000 µg	cyproterone acetate

Therefore, the results presented are based on a sample of 20 healthy women (10 NC and 10 HC users). The majority of the women were Caucasian (6 NC and 8 HC user) and the rest had an Arab (3 NC and 1 HC user) or Asian (1 NC) ethnic background. Table 4.2 presents the

physical characteristics and the PAI of the participants in the two groups. There were no significant differences in any of these variables between the two groups.

Table 4.2. Baseline characteristics of the NC and HC groups.

	NC (n 10)	HC (n 10)	p
Age (y)	26.9 ± 6.2	23.5 ± 4.3	0.171
BW (kg)	63.6 ± 11.0	57.8 ± 9.3	0.216
Height (m)	1.68 ± 0.09	1.66 ± 0.06	0.547
BMI (kg/m ²)	22.5 ± 2.2	21.0 ± 2.4	0.159
FM (kg)	18.7 ± 6.0	16.0 ± 7.3	0.382
FM (%)	28.6 ± 5.5	26.9 ± 8.3	0.579
FFM (kg)	41.5 ± 3.7	41.5 ± 3.7	0.108
Waist (cm)	72.2 ± 6.1	67.9 ± 4.5	0.090
Hip (cm)	99.8 ± 7.1	96.1 ± 8.0	0.279
Waist-to-hip ratio	0.72 ± 0.04	0.71 ± 0.04	0.429
PAI	8.4 ± 1.4	7.9 ± 1.1	0.420

Means ± SD. BW, body weight; FM, fat mass; FFM, fat free mass; PAI, physical activity

According to the Premenstrual Syndrome Screening Tool (PSST) (Steiner et al 2003), none of the participants suffered from Premenstrual Dysphoric Disorder. However, seven women displayed PMS according to the PSST (2 NC and 5 HC users).

4.3.2. RMR

As an average of the MC, RMR was 1656 ± 88 and 1579 ± 49 kcal/d (6929 ± 368 and 6606 ± 203 kJ/d) for NC and HC groups, respectively, with no significant differences between groups. Relative to BW, this corresponded to 26.3 ± 0.7 and 27.8 ± 0.9 kcal/kg/d for NC and HC groups, respectively.

The inter-individual variation (CV%) in RMR in NC and HC group was 15.9% and 9.7%, respectively. When accounting for differences in BW, the inter-individual variation in RMR/kg/d was 7.8% CV and 10.0% CV in NC and HC group, respectively.

In the NC group, although RMR was higher in the LPh than the MPh and the FPh (mean difference: 45 ± 142 and 91 ± 94 kcal/d, respectively), the differences across the three phases of MC were not significant ($p = 0.122$) (Table 4.3). Similarly, no significant differences in RMR were found between the ON and OFF stages in the HC group ($p = 0.971$) (Table 4.4).

Table 4.3. RMR at the different phases of the MC in the NC group.

	MPh	FPh	LPh	p
RMR (kcal/d)	1649 ± 100	1603 ± 80	1695 ± 80	0.122
RMR (kcal/kg/d)	26.1 ± 0.8	25.6 ± 0.8	26.9 ± 0.6	0.089
RMR high CV (kcal/d)	1727 ± 194	1625 ± 151	1800 ± 159	0.108
RMR low CV (kcal/d)	1571 ± 64	1582 ± 76	1589 ± 60	0.564

Means ± SE. *High CV* ≥5%; *low CV* <5%

Table 4.4. RMR at the different stages of the MC in the HC group.

	ON	OFF	p
RMR (kcal/d)	1583 ± 51	1574 ± 47	0.711
RMR (kcal/kg/d)	27.8 ± 0.9	27.9 ± 1.1	0.953

Means ± SE. *ON*, stage taking the HC; *OFF*, stage not taking the HC

Similar intra-individual variation in RMR was observed in the NC and HC groups, i.e. $6.5 \pm 0.9\%$ CV vs. $5.3 \pm 0.3\%$ CV, respectively ($p = 0.453$). However, the CV in the NC group ranged between 3.0-11.7%, whereas a narrower CV range was observed in the HC group (3.3-6.7%). In fact, when subdividing the NC group into high ($\geq 5\%$) and low ($< 5\%$) CV (in agreement with Henry et al (2003)), a distinctive pattern in RMR was displayed, i.e. with a clear rise in the LPh of the high CV participants ($n = 5$) and a steady plateau in the low CV group ($n = 5$) (Fig 4.1). The difference between the LPh and the FPh in the high CV group increased to 176 kcal/d (734 kJ/d), however, it was not significant ($p = 0.156$). Women in the low CV NC group only showed an averaged difference of 7 kcal/d (29 kJ/d) between these two phases (see Fig 4.2. for individual differences between phases in the high and low CV NC women).

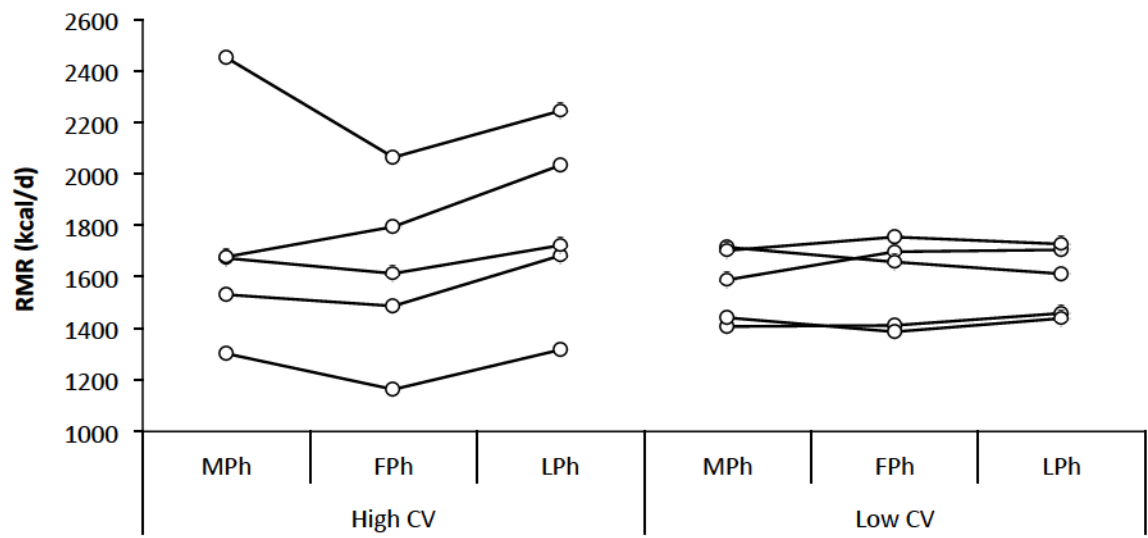


Fig 4.1. Averaged RMR (kcal/d) per individual and phase in the high CV and low CV groups of NC women

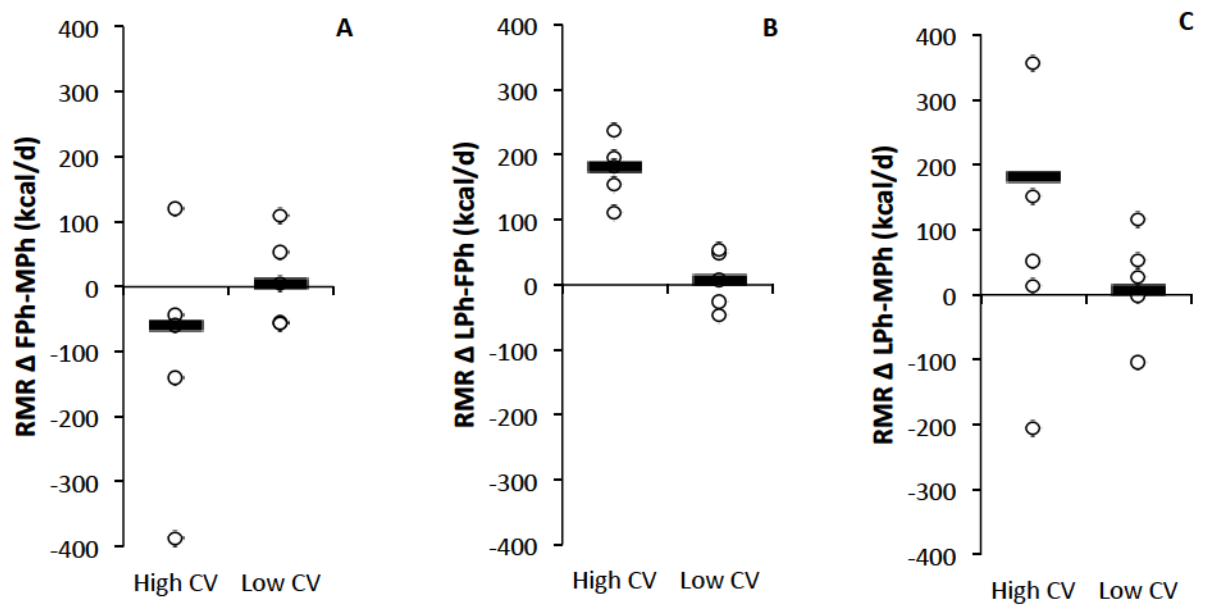


Fig 4.2. RMR difference (Δ) between phases of the MC (kcal/d) calculated as (A) $FPh - MPh$, (B) $LPh - FPh$ and (C) $LPh - MPh$. (■) are medians of the differences in RMR between phases.

4.3.3. Ovarian hormones

In the NC group, all participants successfully ovulated during their MC as reached P_4 concentrations were > 5 ng/ml during the LPh (Piers et al 1995). Plasma E_2 levels were significantly higher in the FPh and LPh compared to the MPh, whereas P_4 levels were significantly greater in the LPh than the MPh and FPh ($p < 0.001$ all) (Table 4.5). Although overall salivary hormones followed the same pattern, pairwise comparisons between salivary E_2 levels did not reach statistical significance (Table 4.5).

Table 4.5. Ovarian hormone levels in plasma and saliva at the different phases of the MC in the NC group

	MPh	FPh	LPh	<i>p</i>
Plasma E_2 (pg/ml)	45.47 \pm 6.53 ^{a b}	176.39 \pm 2.29	174.28 \pm 2.22	<i><0.001</i>
Plasma P_4 (ng/ml)	0.86 \pm 0.05 ^a	0.84 \pm 0.09 ^a	11.65 \pm 1.09	<i><0.001</i>
Salivary E_2 (pg/ml)	2.21 \pm 0.23	2.79 \pm 0.15	2.75 \pm 0.17	<i>0.046</i>
Salivary P_4 (pg/ml)	87.58 \pm 17.57 ^a	84.22 \pm 20.64 ^a	201.23 \pm 35.46	<i><0.001</i>

Means \pm SE. ^a Significantly different to the LPh. ^b Significantly different to the FPh.

In the HC group, plasma E_2 levels in three participants were below the detection limit (<5 pg/ml), thus their data was removed from this part of the analyses. There were no differences in plasma E_2 ($n = 7$) and P_4 ($n = 10$) between the two stages of the MC in the HC group, despite a strong tendency for greater E_2 levels in the OFF stage compared to the ON stage (39.60 ± 6.63 vs. 18.03 ± 5.12 pg/ml) ($z = -1.859$, $p = 0.063$). No differences were found between stages in salivary concentrations for either hormone.

Plasma E_2 and P_4 levels as an average of the cycle were higher in the NC group compared to the HC group (153.24 ± 8.92 vs. 24.42 ± 3.70 pg/ml and 5.46 ± 0.55 vs. 0.53 ± 0.08 ng/ml, respectively) ($p < 0.001$). No significant differences were found in salivary concentrations for either hormone between groups.

4.3.4. RMR and ovarian hormones in the LPh

There were no correlations between plasma P_4 or E_2 and absolute RMR (kcal/d) or relative (kcal/kg/d) RMR in the LPh (Table 4.6). However, there was a significant negative correlation between absolute RMR and P_4 levels in saliva ($r = -0.467$ $p = 0.002$) as well as in the ratio of P_4 levels between specimens (i.e. saliva:plasma) ($r = -0.404$ $p = 0.009$). Moreover, there was a

positive correlation between absolute RMR and the ratio of the two hormones ($E_2:P_4$) in saliva and plasma showed (Table 4.6). When RMR was controlled by BW, no correlations were observed with the ovarian hormones in saliva or plasma (Table 4.6).

Table 4.6. Correlations between RMR (kcal/d and kcal/kg/d) and hormone levels and body composition in the LPh

RMR	E_2 (pl)	P_4 (pl)	$E_2:P_4$ (pl)	E_2 (sa)	P_4 (sa)	$E_2:P_4$ (sa)	E_2 sa:pl	P_4 sa:pl	BW	FFM	FM	%FM
kcal/d	<i>r</i>	0.197	-0.142	0.307	-0.078	0.381	-0.241	-0.404	0.733	0.576	0.364	0.546
	<i>p</i>	0.174	0.330	0.038	0.610	0.002	0.115	0.009	<0.0001	<0.0001	0.009	<0.0001
kcal/kg/d	<i>r</i>	-0.033	0.164	-0.216	0.103	-0.217	0.167	-0.059	-0.311	0.157	-0.451	-0.460
	<i>p</i>	0.825	0.259	0.149	0.500	0.193	0.280	0.716	0.028	0.276	0.001	0.008

Pl, plasma; *sa*, saliva. **Bold** indicates significant correlation ($p < 0.05$)

In order to better understand the potential role of P_4 levels in RMR and its variability, correlations between this hormone and other parameters were made (Table 4.7).

Table 4.7. Correlations between P_4 levels and body composition in the LPh.

P_4		BW	FFM	FM	%FM
Saliva	<i>r</i>	-.572	-.267	-.482	-.545
	<i>p</i>	<0.0001	0.084	0.001	<0.0001
Plasma	<i>r</i>	-0.319	-0.288	-0.076	-0.180
	<i>p</i>	0.022	0.041	0.594	0.207

Bold indicates significant correlation ($p < 0.05$)

Regarding the different models that explain RMR, we found that there was no model that used the ovarian hormones to explain the variance in RMR which could be accepted (i.e. was not violating any of the statistical assumptions) when pooling all the data points of all the participants (NC and HC users). Thus we subsequently ran the models in the NC group and only when the LPh was selected. A multiple linear regression model that included BW, FM, FFM, and E_2 and P_4 ratios between specimens revealed that BW, %FM and salivary to plasma P_4 levels could significantly explain 76% of the variance in RMR in that phase of the MC ($p = < 0.001$). BW and %FM together explained 73% of the variance, thus the ratio in P_4 between specimens added 3 points to the variance, with all the assumptions of a multiple regression (e.g. homoscedasticity, multicollinearity) taken into account.

4.3.5. Substrate metabolism

The respiratory exchange ratio (RER) of the MC was similar between groups (0.851 ± 0.012 vs. 0.871 ± 0.017 for NC and HC groups, respectively). Likewise, substrate oxidation rates and contribution to energy spent did not differ significantly between groups (Table 4.8).

Table 4.8. Substrate oxidation rates (g/min) and contribution (%) in the MC of the NC and HC groups

	NC (<i>n</i> 10)	HC (<i>n</i> 10)	<i>p</i>
Fat oxidation rates (g/min)	0.062 ± 0.007	0.050 ± 0.006	0.458
CHO oxidation rates (g/min)	0.152 ± 0.011	0.168 ± 0.017	0.437
Fat contribution (%)	47 ± 4	41 ± 5	0.205
CHO contribution (%)	53 ± 4	59 ± 5	0.205

Means \pm SE. CHO, carbohydrate

Moreover no differences were observed in RER or substrate oxidation rates between phases of the MC, nor between ON and OFF stages (Table 4.9).

Table 4.9. Substrate oxidation rates in the different phases of MC in the NC and HC groups

	NC (<i>n</i> 10)			<i>p</i>	HC (<i>n</i> 10)		<i>p</i>
	MPh	FPh	LPh		ON	OFF	
RER	0.842 ± 0.014	0.858 ± 0.012	0.851 ± 0.014	0.451	0.890 ± 0.009	0.893 ± 0.019	0.849
Fat (g/min)	0.064 ± 0.024	0.058 ± 0.021	0.063 ± 0.026	0.495	0.050 ± 0.006	0.053 ± 0.009	0.959
CHO (g/min)	0.145 ± 0.014	0.151 ± 0.010	0.157 ± 0.012	0.670	0.169 ± 0.016	0.165 ± 0.022	0.796

Means ± SE CHO, carbohydrate

Similarly, no significant differences were found in substrate contribution to energy expenditure between phases of the MC nor between ON and OFF weeks (Fig 4.3).

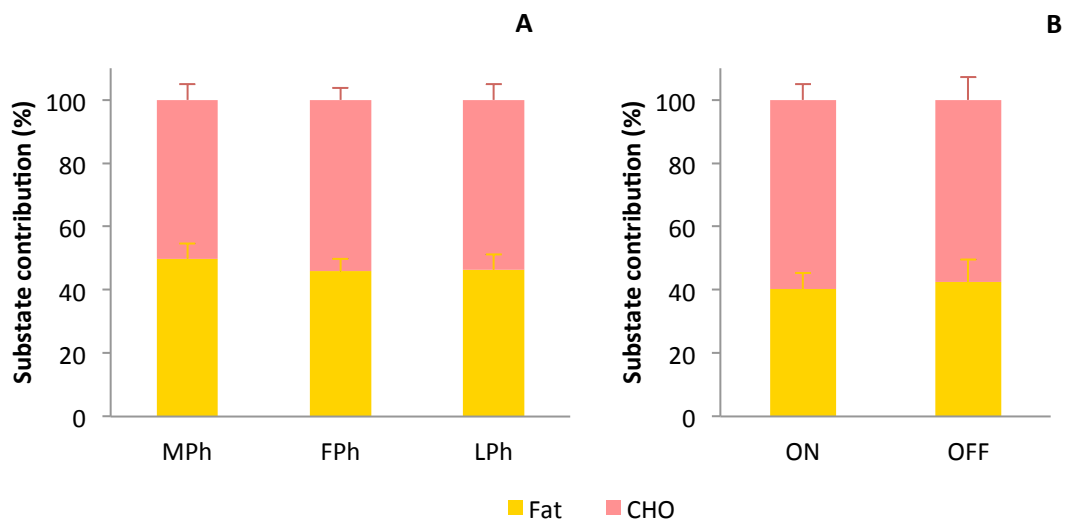


Fig 4.3. Substrate contribution (%) to energy expenditure during the phases of the MC in the NC group (A) and in the ON and OFF stages of the HC group (B).

4.3.6. RMRp vs. RMRm

RMRp underestimated RMRm by 14% (260 ± 22 kcal/d i.e. 1088 ± 21 kJ/d) as an average of all predictive equations with <50% of the whole sample of women having an accurate RMRp (Table 4.10). When comparing the reliability of the equations between groups, the results in terms of bias (i.e. $RMRm - RMRp$) showed that both groups produced an error of the same magnitude (14%) with a similar prevalence of accurate predictions (37% and 32% for NC and HC groups, respectively).

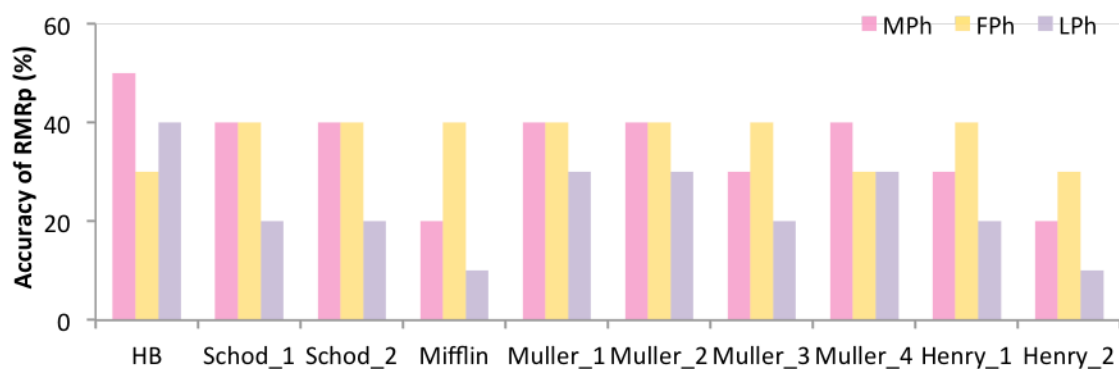
Table 4.10. Mean bias (\pm SD) and accurate predictions (%) of the predictive equations from RMRm in the MC.

Predictive equation	Bias (%)	Accuracy (%)
Harris Benedict	11 \pm 10	40
Schodfield_1	14 \pm 10	40
Schodfield_2	14 \pm 10	40
Mifflin	15 \pm 8	30
Muller_1	15 \pm 9	40
Muller_2	13 \pm 9	40
Muller_3	16 \pm 9	40
Muller_4	14 \pm 10	40
Henry_1	16 \pm 9	30
Henry_2	16 \pm 9	30

Regarding the effect that the MC had on RMRp, a magnified error was observed in the LPh as the bias (Table 4.11) and the prevalence of inaccurate predictions (Fig 4.4) increased in the NC group.

Table 4.11. Bias (%) of the predictive equations from RMRm as an average of the phases of the MC in the NC group (means \pm SD).

Predictive equation	MPh (%)	FPh (%)	LPh (%)
Harris Benedict	11 \pm 11	8 \pm 11	13 \pm 9
Schodfield_1	14 \pm 11	11 \pm 11	16 \pm 9
Schodfield_2	13 \pm 11	11 \pm 10	16 \pm 9
Mifflin	14 \pm 9	12 \pm 9	17 \pm 7
Muller_1	14 \pm 10	12 \pm 10	17 \pm 8
Muller_2	13 \pm 10	11 \pm 10	19 \pm 11
Muller_3	15 \pm 10	13 \pm 10	18 \pm 8
Muller_4	13 \pm 11	11 \pm 12	16 \pm 9
Henry_1	16 \pm 11	14 \pm 10	18 \pm 8
Henry_2	15 \pm 10	13 \pm 10	18 \pm 7

**Fig 4.4.** Accuracy of RMRp in the different phases of the MC for the ten predictive equations in the NC group. *HB*, Harris Benedict; *Schod*, Schodfield.

An example of the absolute bias in RMRp across the phases of the MC in the NC group using the Henry_1 equation is shown in Fig 4.5.

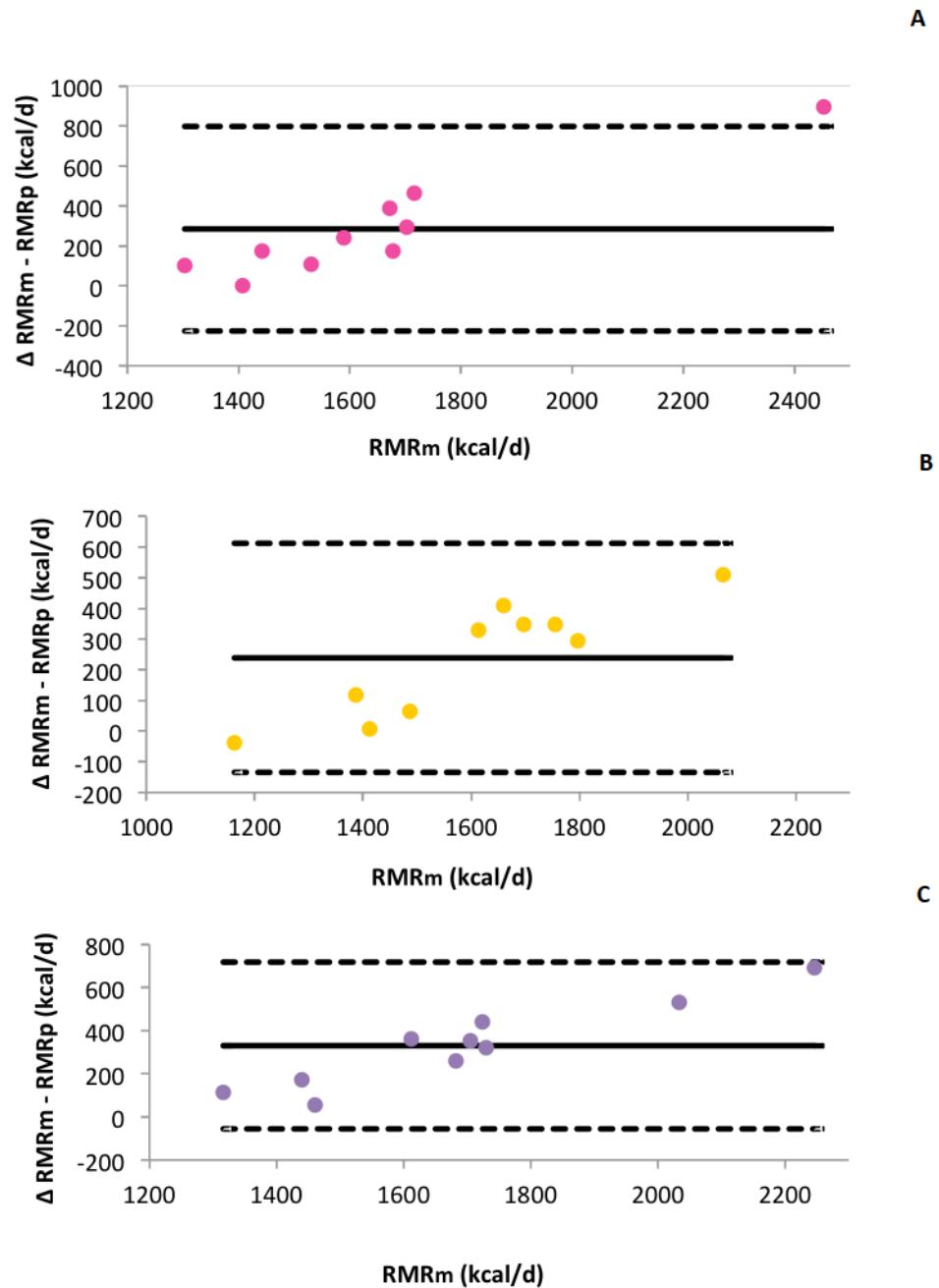


Fig 4.5. Difference between RMRm and RMRp predicted by the Henry_1 equation against RMRm in the MPh (A), FPh (B) and LPh (C).

In the HC group, there were no differences between weeks ON and OFF in the bias or the accuracy in any of the equations except for the Harris Benedict where accurate predictions improved in the ON week (60 vs. 40 %).

4.4. Discussion

The aim of this study was to investigate whether RMR is significantly increased in the LPh compared to the MPh and the FPh in women who have natural and regular MCs as a result of a higher day-to-day variability in their RMR compared to men and women taking HC. This research is of importance to have a better understanding of women's energy metabolism as it is well known that RMR is the predominant constituent of total daily energy expenditure. Having an accurate measurement of the RMR is crucial to estimate energy requirements in light of the current increasing trend of overweight and obese females (WHO 2015) as well as all the comorbidities associated with energy balance.

4.4.1. RMR throughout the MC

The present investigation showed that there were no significant differences in RMR across the phases of the MC in a group of ten healthy NC women and in the different stages of a MC and in ten women using combined HC. This suggests that, in general, women are as effective as men in maintaining their RMR constant and therefore changes in energy balance and BW and composition may come from other variables of the energy balance equation, e.g. EI, PA. This is in agreement with some (Horton et al 2002, Howe et al 1993, Piers et al 1995) but not all previous research as some studies have shown that RMR increases in the LPh compared to the FPh (Curtis, Henry, Birch, et al 1996, Matsuo et al 1999, Solomon et al 1982).

The reasons behind these discrepancies might be related to the different methods employed in the studies. In fact, to our knowledge this is the first study that averages RMR along with the ovarian hormone levels for more than two days in each phase of the MC (except for the MPh due to its short length). This is of relevance not only because previous significant variations found could be stimulated by other uncontrolled factors, e.g. variability of the indirect calorimeter, participant not stable during a measurement, meal standardization of the night before, but also because significant differences in RMR would need to be sustained for more than one day to induce any physiological effect on the energy balance equation provided that all other factors, such as PA and energy intake, are constant. Therefore, having a more accurate picture of RMR in the different phases of the MC, by repeating measures more than twice in each phase, could confirm whether such potential variations are metabolically meaningful in terms of frequency (i.e. number of different RMR values) and magnitude (i.e. difference between RMR measurements).

Another common drawback of some previous research was to divide the MC by changes in body temperature or by counting retrospectively since the start of the MPh. These two methods present clear disadvantages as they have been found to be not accurate in all women due to the large variability in the length of the cycle and its phases and the unavailability of finding a clear temperature change on the ovulation day (Fehring et al 2006, Guida et al 1999). Furthermore, depending on the study design, the phases of the MC were differently defined in terms of number and the criteria of classifying each phase. For instance, depending on the study there were two, three or even four phases divided by pre- and post-ovulation or by early and late FPh and LPh. Therefore, this becomes challenging not only when comparing results between studies, but also when determining the accuracy of the findings presented. Therefore, our study design should have been able to precisely obtain any representative changes in RMR amongst phases of the MC, provided that a big enough sample size was used. Nevertheless, our findings suggested that, despite reaching a 91 kcal averaged difference between the LPh and the FPh, this was not statistically significant. It would be inappropriate to ignore the possibility that the lack of statistical difference was due to an insufficient sample size, thus, our results need to be carefully interpreted.

4.4.2. RMR variability

The present study showed an intra-individual variation in RMR of 3.0-11.7% in NC women. This is broadly consistent with earlier findings suggesting a wider variability in the CV of RMR within NC women than men (Curtis, Henry, Birch, et al 1996, Henry et al 2003, Piers et al 1995, Solomon et al 1982). This suggests that a single measurement of RMR might depict an erroneous starting point for the calculation of daily energy requirements in several women whose RMR is so variable. Indeed, there was an average of 111-226 kcal/d (464-991 kJ/d) (7-14%) increase in the LPh compared to the FPh in the group of women with a high CV within the NC group. It has been estimated that a daily 100 kcal (418 kJ) deficit (achieved by greater energy expenditure and / or lower energy intake) can lead to a decrease in BW overtime (James O Hill et al 2003). Thus, the increase in RMR from the FPh to the LPh in some NC women, namely in those with high CV in RMR, might be of clinical and physiological relevance despite the insufficient statistical significance. On the other hand, participants with a low CV in RMR experienced no more than ~50 kcal/d (~223kJ/d) difference between these two phases with no clear direction (i.e. energy deficit or surplus). Less apparent was the effect between the LPh and the MPh assuming that the pattern observed in the high CV group is repeated

cyclically; all expect one participant displayed a carry-over effect as RMR appeared to be reduced from the LPh but not the low levels of the FPh. Therefore, when measuring or estimating energy requirements for a woman, one should take into account that some individuals may experience large differences in caloric needs depending on the MC phase.

4.4.3. Determinants of RMR

The present study showed significant increases in P_4 in the LPh compared to the MPh and the FPh with values reaching the 5 ng/ml limit which is considered the minimum concentration to confirm the existence of ovulation in NC women (Piers et al 1995).

Previous research linked the increase in P_4 in the LPh to an increase in RMR, sleeping metabolic rate (SMR) and 24h-energy expenditure (Matsuo et al 1998, Meijer and Westerterp 1992, Solomon et al 1982, Webb 1986) due to its increasing effect on body temperature (Barton and Wiesner 1945); however, in many studies P_4 levels in blood were not measured thus a direct association between the two could not be obtained and assumptions of ovulatory cycles were made without evidence. This may have therefore, biased the outcome of studies in which P_4 levels might have not increased to the abovementioned threshold (Meijer and Westerterp 1992, Solomon et al 1982, Webb 1986).

Nonetheless, even when P_4 has been successfully detected in urine, energy expenditure (Webb et al. 1986) and increments in SMR (Bisdee et al. 1989) have not always been significantly correlated with pregnandiol-3 α -gucoronide (the urinary metabolite of P_4). Similarly Howe et al. (1993) found that plasma P_4 and E_2 levels of 14 women were not correlated to their RMR in the LPh. This agrees with our findings as no correlations between the ovarian hormones and RMR (absolute or relative) were found.

However, to our knowledge, this is the first study to measure and show a significant correlation between RMR and salivary P_4 . The fact that salivary hormones are significantly and more strongly correlated to RMR than their counterparts in plasma is somehow expected since the first represent the unbound or biologically active form of these hormones. However, having a negative correlation between salivary P_4 and RMR ($r = -0.467$) was unexpected due to the above mentioned thermogenic effect of P_4 (Barton and Wiesner 1945). This association, nonetheless, was supported by the significantly negative correlation of RMR with plasma to saliva ratio of P_4 levels ($r = -0.307$), namely the greater the portion of unbound to bound P_4 , the smaller the RMR in the LPh. Moreover, a positive correlation between salivary $E_2 - P_4$ ratio and

RMR was found, i.e. the greater the proportion of salivary E_2 to P_4 , the higher the measured RMR. This rather contradictory result may be due to the possible interference of other factors, e.g. BW, which cannot be ruled out. In fact, salivary P_4 was negatively correlated to BW, FM and %FM. Even plasma P_4 was negatively correlated to BW. That was corroborated by the disappearance in the association between P_4 levels and adjusted RMR (by BW). Another potential confounding factor that has been suggested in the literature to have a thermogenic effect is norepinephrine, as a β -adrenergic supporter of the sympathetic nervous system (Day et al 2005). Nevertheless, the effect of this catecholamine seems to vary amongst individuals (Day et al 2005) which could partly explain the differentiation between women with a high and a low RMR CV.

Despite not showing a significant and direct relationship between plasma ovarian hormones and RMR, it cannot be rejected that these might still have an effect on RMR. An example of this, was the investigation by Day et al (2005) in which sex hormone concentrations were induced to postmenopausal levels by the intake of an anti-gonadotropin-releasing hormone (GnRH) during six days starting at the FPh of the MC. This resulted in a significant reduction in RMR compared to the LPh and the MPh. Similarly, the physiological decrease in E_2 in postmenopausal women has been linked to a significantly lower RMR ($r = 0.38$, $p < 0.05$) (Lynch et al 2002). However, this may not necessarily indicate a direct effect of the hormones, rather a consequence of the decrease in FFM, which in turn can be associated to the depletion in sex hormones.

Regarding the impact that the measured hormones might have in determining the variance of RMR in the LPh, we found that only the ratio of unbound to bound P_4 could add three points into a model that could explain 76% of the variance of RMR. The 71% of the variance was explained, as expected, by BW and %FM. How BW and body composition have a major impact on RMR has been demonstrated repeatedly throughout the literature (Javed et al 2010, Nelson et al 1992). To our knowledge this is the first study showing a small but significant contribution of the ovarian hormones into RMR. Only Howe et al (1993) found that P_4 had a significant effect on the increase of SMR in the LPh in a group of women in which dietary intake was controlled. Furthermore, Meijer and Westerterp (1992), showed that their regression model predicting SMR improved when adding the MC phase (either pre- or post- ovulation). This model, however, was based from a population of men and women and men were then treated as pre-ovulation phase.

4.4.4. Substrate metabolism

The outcome of this study indicated that the participants were oxidising a higher proportion of calories derived from carbohydrates (CHO) than fat in both groups (53% and 59% vs. 47% and 41% in the NC and HC groups, respectively). This was somewhat surprising because RER was measured at rest and under a 10-12h fasted state, when a fat contribution of >50% is expected (Melzer 2011). A plausible explanation behind this inconsistency could be a high carbohydrate diet or a low training volume (Goedecke et al 2000). Since our sample of participants would not be classified as highly active subjects, it could be hypothesised that their fat metabolism at rest is less predominant than in more trained individuals. Furthermore, McNeill et al (1988) showed how fasting RER could be rapidly changed by diet in an experiment where 11 women were submitted into two different diets containing (1) 45% CHO and 41% fat and (2) 54% CHO and 31% fat for 4 days in each diet. Their results revealed a significant increase in RER from diet (1) to (2) (0.824 and 0.851 for diets (1) and (2), respectively). There was a decrease in fat contribution from 59 to 46% (when protein oxidation is considered negligible). This paper provides evidence of not only how small changes in your diet can have an impact on your substrate metabolism at rest, but also it presented a wide intra-individual variability in RER amongst women (0.790 - 0.890).

The fact that no significant changes were observed in the rate of substrate metabolism across the phases of the MC coincides with past research (Horton et al 2002, Piers et al 1995). Although the results of the present study showed no significant differences in substrate metabolism when comparing the LPh and the FPh, fat oxidation rates were still increased 8% in the LPh which explains the rise in RMR at the end of the MC. In contrast, CHO oxidation rates were only marginally increased (3%) in this phase. These results are in line with Matsuo et al (1998, 1999)'s studies in which RER was reduced in the LPh, indicating a higher fat contribution, but without reaching the statistical significance.

4.4.5. NC vs. HC users

The present study did not find any significant differences in RMR (absolute or relative to BW) between NC women and a group of women taking different formulas of a combined monophasic HC. Similarly, Diffey et al (1997) showed no differences in RMR between NC women and combined HC users. However, when using BW or FFM and FM as covariates, they found that HC users had approximately 5% higher RMR than the NC women. Kimm and

colleagues (2001) also found 50 kcal/d (207 kJ/d) greater in RMR adjusted by race, FFM and FM in a group of young women taking HC than in the controls ($p = 0.04$). Moreover, a longitudinal study showed a rise in RMR by 30kcal/d (151 kJ/d) after the injection of a P₄-only HC compared to RMR during the FPh (Pelkman et al 2001). In contrast, Suh et al. (2003) did not find any differences in energy expenditure at rest before and after the intake of a triphasic HC during 4 months, although that was in a post-absorptive state 3h after consuming a standardised breakfast in a group of moderately active women. Differences between studies may be due to methods used and, more importantly, the type of HC used and the time that these have been used. With regards to the CV% of RMR, the result shown in the present study for the HC group was slightly higher than that observed by Curtis et al (1996) (i.e. 4.5%), and our findings support a wider variability in RMR between individuals who are NC than those who are under HC.

Regarding the substrate metabolism, the present study showed no significant differences between groups although CHO oxidation rates and contribution to total energy expenditure was higher as an average of the cycle and in individual phases in the HC group compared to the NC women. This is in agreement with the longitudinal study by Suh et al (2003) in which CHO contribution to energy expenditure at rest increased from 56% to 66%, albeit not significantly. They also showed a significant increase in insulin levels after using the HC for 4 months, suggesting that glucose flux might be impaired by a defect on the insulin sensitivity as a consequence of using HC. In fact, a cross-sectional study with two groups of sedentary women, one taking HC and the other being in the LPh of their MC (NC) showed how the former displayed a significantly higher postprandial glucose response with 24% higher (not significantly) fasting insulin levels compared to the latter (Jankowski et al 2004). In conjunction these studies indicate the possibility that substrate metabolism might be indeed affected by HC but significant results might have not been attained because of the different composition of the HC, the characteristics of the participants (active vs. sedentary) and the small sample size of the studies.

As expected, HC users presented lower ovarian hormone levels than their counterparts due to the suppressing effect that the estrogens and progestins exert on the FSH, thus impairing the ovary activity and the production of endogenous sex steroid hormones (Frye 2006). The fact that E₂ levels were not detectable in blood in three of the HC users is not completely unforeseen as although the concentrations of ethinylestradiol were similar between the different drugs, i.e. 20-35 µg, ethinylestradiol has been shown to have diverse

pharmacokinetics that affects the absorption, metabolism, bioavailability and elimination differently between races, cycles and individuals (Goldzieher and Stanczyk 2008).

4.4.6. RMRp vs. RMRm

Our results showed that ten validated equations that predict RMR in adults were not appropriate in estimating RMR in a group of 20 (mainly) Caucasian not obese women of a fertile-age. Not only because less than half of the sample had inaccurate predictions with any of the ten equations, but also because the average bias was of -260 kcal/d (~1088 kJ/d). That is concerning because the majority of nutrition practices do not possess the means by which RMR can be measured, thus they rely on estimations from equations such as those used in the present study.

Nevertheless these results need to be interpreted cautiously since the usual sample size of studies looking at predictive equations of RMR is larger (50-100 subjects). The reader needs to be reminded though, that the primary purpose of this investigation was to look at the variability in RMRm in women throughout a MC which requires more than one visit (approximately 12) to the laboratory per participant thus resulting impractical for a study that aims to determine the reliability of predictive equations. However, the authors valued its input to the literature since to our knowledge no others have investigated this matter. Moreover, the fact that RMRm was an average of different days rather than a snapshot of one visit might improve the accuracy of the measurement per se.

Combining bias and accuracy of each equation, it could be suggested that the Harris Benedict equation was the most reliable of the ten and that the Henry equations, especially the number 1, were the weakest in predicting RMR for our sample. However, it would not be sensible to prioritise any particular equation of the ten since their accuracy rate may be considered poor. This disagrees with a recent study by Weijs and Vansant (2010) in which 536 Dutch women of a wide range of BMI, showed to have a bias of -1.8% (with Mifflin RMRp) to 3.0% (with Muller_1) and an accuracy of 59% (with Schodfield_1) to 70% (with Muller_3). This reliability was maintained or improved when looking at the non-obese subset of their sample, therefore other reasons might explain these differences. Firstly, their population could be any women of >18y which included post-menopausal women and secondly they were using another indirect calorimeter. Regarding the second concern, a recent study (Kennedy et al 2014) demonstrated that RMR measured under our indirect calorimeter (i.e. the GEM) was significantly higher than

the Deltatracc which was commonly used in the past and is no longer available (Allen et al 2000, Diffey et al 1997, Van Pelt et al 1997). This may explain, in part, the consistent underestimation of RMRp; nonetheless, the authors suggested that the GEM was a reliable instrument for the measurement of repeated readings, thus making it appropriate for the main purpose of our study. Furthermore, Kennedy et al (2014) questioned the validity of the Deltatracc as an accurate comparison due to the poor available maintenance of such an old instrument.

The fact the RMRp was more biased in the LPh was not surprising once recognised that (a) the error was an underestimation and (b) RMRm tended to increase in that phase of the MC. Finally, the higher relative number of accurate predictions in the HC group seemed to respond to a small sample size artefact rather than a meaningful outcome.

4.4.7. Limitations

The main limiting factor of our findings is that the sample size obtained might have not been sufficiently large to show significant differences in RMR across the phases of the MC. Indeed the retrospective analyses to calculate sample size showed that a total of 21 NC women would have been required to study RMR throughout the phases of the MC. Nevertheless, the initial estimation based on previous studies was of a smaller size and this limitation might be blunted by the observed inter-variability between participants, e.g. high and low CV in RMR. This was restricted by practical limitations related to the difficulty of the testing protocol involved for each participant, i.e. many visits to the laboratory in specific times and the use of invasive techniques such as the venepuncture. Another limitation of the study was that participants in the HC group were using different HC prescribed by their GP. Nevertheless, the researcher ensured that all HC users were under the same type of HC, i.e. combined and monophasic, to minimize differences between individuals. Moreover, a within-subject design was used to compare the RMR at the different stages of the MC

Having all the participants starting in the same phase of the MC might have had a learning/adapting effect under the ventilated hood indirect calorimeter. Nevertheless, the trend towards an increase rather than a reduction in RMR at the end of the cycle, suggests that an adaptation to the hood throughout the days did not occur and that the initial 10 min under the hood before the measurements were enough for the participants to be rested during the data collection. Furthermore, having participants starting at the other phases would have just increased the difficulty for the researcher from a practical point of view as MC phases would

require to be defined before the ovarian hormones were measured or phases of different MC would have been needed.

Finally, other parameters such as testosterone, epinephrine and noradrenaline that could have confounded or explained better the influence of the ovarian hormones on RMR could not be measured. These have been associated with changes in energy and substrate regulation (Mauras et al 1998, Shi et al 2009) and they might also fluctuate within the MC (Matsuo et al 1998, Rothman et al 2011).

4.5. Conclusion

To conclude, the present investigation examined RMR across the phases of the MC in a group of NC women and HC users. This is the first time that the average of more than two days in each phase along with the ovarian levels have been used to explore the variability of RMR in NC women and HC users. Moreover, participants were tested within the same MC using a within-subject design, therefore minimising inter-individual and inter-cycle differences compared to previous studies. This study has found that in the population studied, there were no significant variations in RMR in NC women and HC users throughout their MC. However, some individuals presented clinically meaningful changes in their RMR which confirmed the high inter-individual variability amongst women and highlights the need of personalisation in the design and prescription of diets in this population. Moreover, this is the first study to show a negative correlation between RMR and salivary P_4 as well as the ratio between plasma and saliva P_4 during the LPh. Nevertheless, these associations disappear when RMR is adjusted with BW. Finally, the ratio between saliva and plasma for P_4 levels contributed in the variance of RMR in the LPh in a model where BW and %FM explained the majority of RMR variance.

Further research needs to be done to understand the possible underlying mechanisms why some NC women may be more likely to experience substantial changes in their RMR and what are the consequences in the long term. Namely, why are there women with a high and a low intra-variation in their RMR and is there any long-term metabolic benefit in having a high or a low variability. Since both groups of NC women showed to maintain their BW, it could be hypothesised that women in the high CV group need to unconsciously compensate their energy requirements by changing their EI to maintain their BW, thus potentially making them more efficient in adapting to acute changes in energy balance in the long term.

5. Appetite responses during the MC (Study 3)

5.1. Background

It is well known that the process of digesting food involves numerous actions by different organs in order to prepare food for its absorption in the intestine. Once the bolus reaches the stomach, this will be held to be mixed with the gastric juices to form the chyme and be broken down into particles small enough to go through the pyloric sphincter (Smolin and Grosvenor 1994). The rate at which the chyme passes into the small intestine and the amount of time required to fully empty the stomach is dependent on the characteristics of the meal, i.e. volume, energy density, weight, acidity (Malagelada and Azpiroz 2010).

Nonetheless, this is ultimately regulated by different gastric and intestinal hormones (e.g. gastrin, CCK, GLP-1) that will ensure the availability of the intestine to continue the digestive and absorptive process (Smolin and Grosvenor 1994). PYY is one of the multiple regulators of the digestion process and its main role is to mediate the ileal brake, i.e. the delay in the transit of the chyme through the gastrointestinal tract (Onaga et al 2002), that results in an increase in satiety. PYY's secretion in the distal intestine is stimulated post-prandially and this is related to the caloric and macronutrient content of the meal (Adrian et al 1985, Batterham et al 2003).

Multiple studies have shown how changes in GE and PYY response to a meal-test can have an impact on the appetite sensations and the subsequent food intake (Clegg and Shafat 2010, Stoeckel et al 2008). Nevertheless, many of the studies conducted in this area avoid the participation of women or control their protocol by testing women in a specific phase of the MC, as it is generally accepted that women can experience changes in their habitual food intake upon the phase of their MC (McNeil and Doucet 2012). These changes seem to result from a bigger meal size (rather than from an increased number of meals) in the LPh than the FPh (Asarian and Geary 2013). Therefore, it could be suggested that women may experience changes in their food intake due to fluctuations experienced primarily in their satiation (the process of finishing meal), rather than their satiety (the process inhibiting the start of a meal), throughout the MC. Furthermore, in light of the global higher obesity prevalence in women than men (WHO 2015), having a better understanding on women's appetite physiology seems imperative.

The objective of this study was to investigate the appetite response across the phases of the MC. Precisely we aimed to study whether eating the same breakfast in each phase of the MC would change the GE rate, the PYY response and the satiety feelings of the meal to ultimately

have an impact on the food intake of a buffet lunch served four hours later. Furthermore, the aim was to associate any potential differences to the naturally occurring fluctuations in E_2 and P_4 of the MC. We secondly aimed to investigate whether food intake measured during three days for each MC phase changes significantly and whether food intake on the days under the free-living conditions was affected by the MC phase.

5.2. Methods

5.2.1. Protocol

Once the participant was accepted to partake in the study, she was given a fertility monitor (Clearblue) to assist in the scheduling of her visits to the laboratory based on the three different MC phases i.e. MPh, FPh and LPh. The three chosen days were aimed to display a very distinguishable profile in the ovarian hormones: MPh, E_2 and P_4 at low concentrations; FPh, E_2 at high concentrations while P_4 remains low; and LPh, E_2 and P_4 at high concentrations. The MPh visit was scheduled as soon as the participant notified the start of a new MC and this was performed within 4 days of starting the MC. From day 6 of the MC, participants tested their morning urine using the fertility monitor to measure their E3G and LH levels. The testing days for the FPh and LPh were scheduled based on each individual's MC profile and the fertility monitor readings (see more details in chapter 2.11).

Once the visit to the laboratory was scheduled, participants were also asked to record their food intake for three days in each MC phase: (1) the day before coming to the laboratory, (2) the testing day and (3) the day after the visit to the laboratory. (See Fig 5.1 for an example testing timeline within a MC). Moreover, they were asked to wear a body monitoring system to estimate their PA levels to be able to validate their EI from the food diary and detect any potential misreporting.

In the evening before each test day, participants were asked to avoid the consumption of caffeine and alcohol and the practice of any unusual strenuous exercise that they would not usually do as part of their normal 'daily lifestyle'.

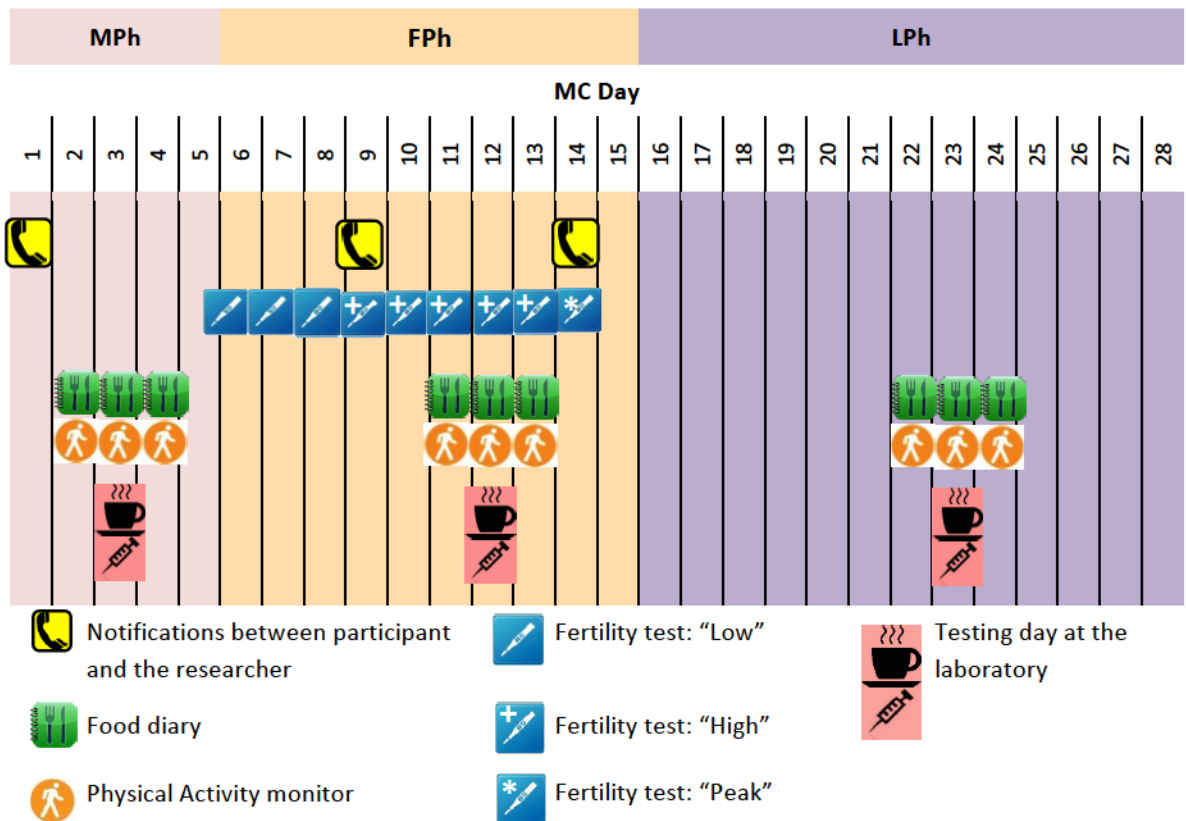


Fig 5.1. Example experiment time line during a MC.

During the visits to the laboratory participants were requested to arrive between 7:00-9:30h to get their body composition assessed and then a cannula was inserted into their arm to obtain the baseline blood sample ($t = 0$). Immediately after, they filled in the VAS for appetite sensations and the first breath sample for measurement of GE was collected. Then the participant was taken to the kitchen to consume the standardised breakfast labelled with ^{13}C octanoic acid which included scrambled eggs on toast, pineapple and a drink of their choice (water, coffee or tea with/out milk and sugar). Participants were asked to finish their breakfast within 15min. As soon as they finished their breakfast, they were taken straight away to the lab to collect the first post-ingestion breath sample, blood sample and satiety scores. Subsequent breath samples were taken every 15 min until min 240. Subsequent blood samples and satiety scores were taken every 15 min until $t = 60$ thereafter every 30 min until $t = 240$.

Each blood measurement required only 4ml to quantify the total PYY concentrations (except at baseline when two tubes were used to measure the ovarian hormones as well, i.e. 8ml), therefore a total of 48ml of blood in 11 time points were collected over 4 hours. See flow chart for clarification of the protocol (Fig 5.2).

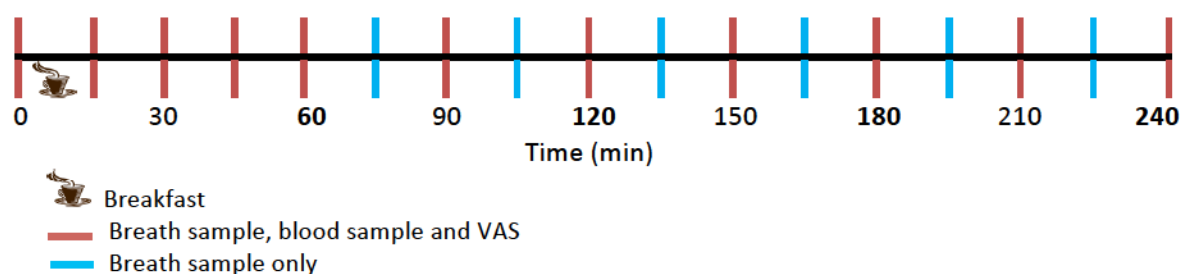


Fig 5.2. Timeline of events during each of the testing days in the laboratory.

Immediately after the last blood sample, the cannula was removed and the participant was taken to the kitchen for an *ad-libitum* lunch buffet (see details of foods included and the nutritional composition in chapter 2.8.2). Participants were invited to eat until comfortably full and following that they were free to leave. Lunch intake was calculated by subtracting the weight of the food left from that of the food prepared for the buffet and intakes of energy, carbohydrates, sugars, protein, fats, saturated fat, fibre and sodium were determined according to the food's label.

Blood samples were centrifuged to extract their plasma; this was then stored at -80°C for future analyses by ECLIA for E_2 and P_4 and ELISA for total PYY (see chapter 2.4.2). Averaged intra-duplicates CV for PYY ELISA assays was $6.26 \pm 1.40\%$. Breath samples were analysed using an isotope ratio mass spectrometer to quantify the excess amount of labelled oxidised octanoic acid (i.e. $^{13}\text{CO}_2$) above baseline for each time point. This was expressed as the percentage of dose recovered per hour and this was fitted into a non-linear regression model (Ghoos et al 1993) (see chapter 2.7.3 for more details). Food intake recorded by food diaries was measured by the use of a nutrition analyses software (Nutritics V3.74 Professional Edition) and intakes of energy, carbohydrates, sugars, protein, fats, saturated fat, fibre and sodium were determined per day and per phase of the MC for each participant. Physical activity levels from the activity monitor were averaged across the nine days and then compared to the EI reported from the food diaries.

5.2.2. Calculations and statistical analyses

PYY peak was defined as the highest PYY concentrations achieved post-baseline. Concentrations of PYY and VAS scores were used to calculate their total AUC using the trapezoidal method.

From the curve created by the percentage of dose recovered per hour of the breath samples model, different GE parameters could be calculated using Ghooos et al (1993) and Schommartz et al (1997)'s equations (see chapter 2.7.3 for more details).

One-way repeated measures ANOVA or Friedman test was used to test differences across the phases of the MC for PYY AUCs at every hour, ovarian hormone levels, GE parameters and food intake across the phases of the MC. When significant differences were found, a Bonferroni post-hoc pairwise comparison or a Wilcoxon signed-rank test was performed, according to the normality of the data.

A 2-way repeated measures ANOVA with time and MC phase as factors was used to analyse PYY levels and PYY change from baseline within subjects as an assessment of the postprandial changes across the MC.

AUC for VAS was analysed with another 2-way repeated measures ANOVA that included the baseline scores as covariates in the analyses.

Associations between EI and PYY, GE and appetite feelings as well as between ovarian hormones and the appetite markers (i.e. EI and PYY, GE and appetite feelings) were analysed by Pearson's or Spearman's correlation, according to the normality of the data.

A sample of nine women was based on the only other study that has looked at appetite hormones responses in the MC (Brennan et al 2009). Retrospective analyses, using the G*Power software with a partial eta square (η^2) of 0.410 and $\alpha = 0.05$, determined that there would have been an 83.4 % chance of correctly rejecting the null hypothesis of no differences in the T_{half} across the phases of the MC with a total of 10 participants. Another retrospective analyses with a partial eta square (η^2) of 0.363 and $\alpha = 0.05$, determined that there would have been an 80.0 % chance of correctly rejecting the null hypothesis of no differences in the PYY AUC at 240 across the phases of the MC with a total of 11 participants.

5.3. Results

5.3.1. Participants characteristics

Fifteen women signed the consent form of which three had to be excluded because of violating the inclusion criteria (i.e. irregular MC and suspicion of suffering Gilbert's syndrome) (see chapter 2.2). Of the twelve remaining women who started the study, two withdrew due to personal reasons and another who completed the study had to be excluded because of unconfirmed ovulation and unavailability to reschedule the LPh testing day. Thus the following results are based on a population of nine NC women (Table 5.1).

Table 5.1. Participants' characteristics at baseline

	Mean \pm SD
Age (years)	31 \pm 6
Height (m)	1.67 \pm 0.09
BW (kg)	63.4 \pm 12.8
BMI (kg/m ²)	22.6 \pm 2.7
FM (%)	29.0 \pm 7.4
FM(kg)	19.1 \pm 7.8
FFM (kg)	44.4 \pm 6.3
Waist-to-hip ratio	0.77 \pm 0.07
PAI	8.26 \pm 0.98

BW, body weight; FM, fat mass; FFM, fat free mass; PAI, physical activity index

5.3.2. MC characteristics and ovarian hormones

Average MC length was 29 ± 3 days. Of the nine participants included, four had a “peak” reading i.e. ovulation was confirmed by the fertility monitor, within their first MC, while three participants only ovulated on the second MC. Averaged “peak” reading happened on day 14 ± 3 of the MC. The two remaining participants attended to their LPh visit at what it was expected to be their mid-LPh. Plasma P₄ levels indicated that these participants had ovulated despite not having a “peak” reading in the fertility monitor. P₄ levels were $>5\text{ng/ml}$ which is considered high enough to have ovulated (Piers et al 1995). Moreover, one of these two participants had a positive LH peak in her personal fertility monitor, thus participants were kept in the study as they seemed to have ovulated despite not having been detected by the fertility monitor.

There were significant differences in E₂ and P₄ concentrations amongst the three phases of the MC ($p < 0.001$ and <0.0001 , respectively) (Fig 5.3). E₂ levels were significantly increased in the FPh and LPh compared to the MPh whereas P₄ levels were significantly higher in the LPh compared to the other two phases.

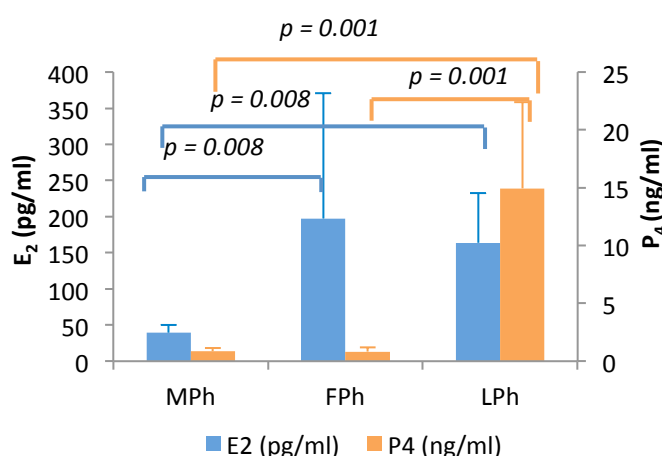


Fig 5.3. E₂ and P₄ concentrations in the different phases of the MC (means \pm SD).

5.3.3. GE

The $^{13}\text{CO}_2$ curve was formed throughout the 4 h after breakfast, ensuring that there was enrichment by the breakfast with ^{13}C . An example of this can be seen in Fig 5.4

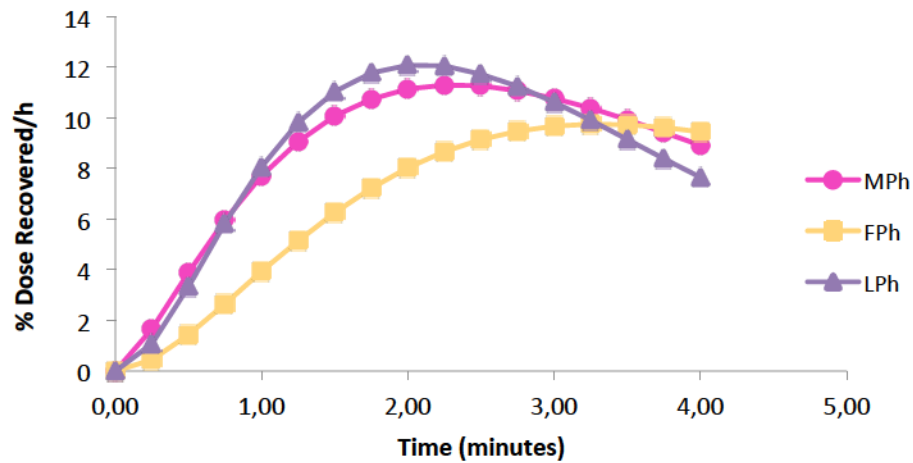


Fig 5.4. Example of a participant's breath $^{13}\text{CO}_2$ curves, expressed in excess atom fraction over the time following each of the three phases of the MC.

There was a significant overall effect of the phase of the MC on T_{half} and T_{asc} (Table 5.2) but none of the specific comparisons between phases indicated a significant difference. However the effects observed seem to mainly reflect that T_{half} was quicker in the LPh compared to the FPh and the MPh (mean difference: 28 ± 31 and 13 ± 15 min, $p = 0.081$ and 0.092 , respectively) and T_{asc} was faster in the LPh compared to the FPh (mean difference: 27 ± 29 min, $p = 0.077$). There was a trend towards a difference in T_{lag} across the phases of the MC ($p = 0.072$). No differences were found in T_{lat} across the phases of the MC.

Table 5.2. GE parameters shown in minutes for MPh, FPh and LPh.

GE parameter (min)	MPh	FPh	LPh	<i>p</i>
T_{half}	101 ± 23	116 ± 46	88 ± 22	0.015
T_{lag}	48 ± 8	51 ± 14	43 ± 12	0.072
T_{lat}	52 ± 7	53 ± 12	48 ± 13	0.264
T_{asc}	128 ± 23	143 ± 41	116 ± 13	0.011
Mean \pm SD				

5.3.4. Total PYY

Due to sampling issues, a total of seven samples (2%) could not be collected. Of these, four were the 150-240 min samples of one participant's FPh, therefore, comparisons from min 150

onwards are only from 8 participants. The three remaining samples (1%) belonged to three different phases, thus concentrations for these time points were estimated as explained in chapter 2.4.3.

PYY levels were significantly different at baseline across the phases of the MC ($p = 0.004$), being significantly lower in the LPh compared to the MPh (59.9 ± 40.5 vs. 91.2 ± 47.5 pg/ml) ($p = 0.008$), but not to the FPh (64.9 ± 9.4 pg/ml, $p = 0.079$). PYY peak was lower in the LPh compared to the MPh and FPh (117 ± 50 vs. 133 ± 47 and 132 ± 48 pg/ml, respectively) but it was not significantly different ($p = 0.264$).

The 2-way ANOVA analyses showed that there was not a significant *phase x time* interaction in PYY levels ($p = 0.169$) (Fig 5.5). Nevertheless, an overall phase effect was shown ($p = 0.041$), yet there were no significant differences between specific phases in total PYY concentrations. However the effects observed seemed to reflect that PYY levels were lower in the LPh compared to the MPh ($p = 0.080$). Furthermore, there was a significant time effect ($p < 0.001$), as PYY levels increased significantly from baseline to $t = 45, 60, 90$ and 120 ($p \leq 0.05$ for all), as well as from post breakfast ($t = 15$ min) to 90 ($p = 0.030$). Finally, there was a significant reduction in PYY concentrations from $t = 180$ to 210 ($p = 0.017$).

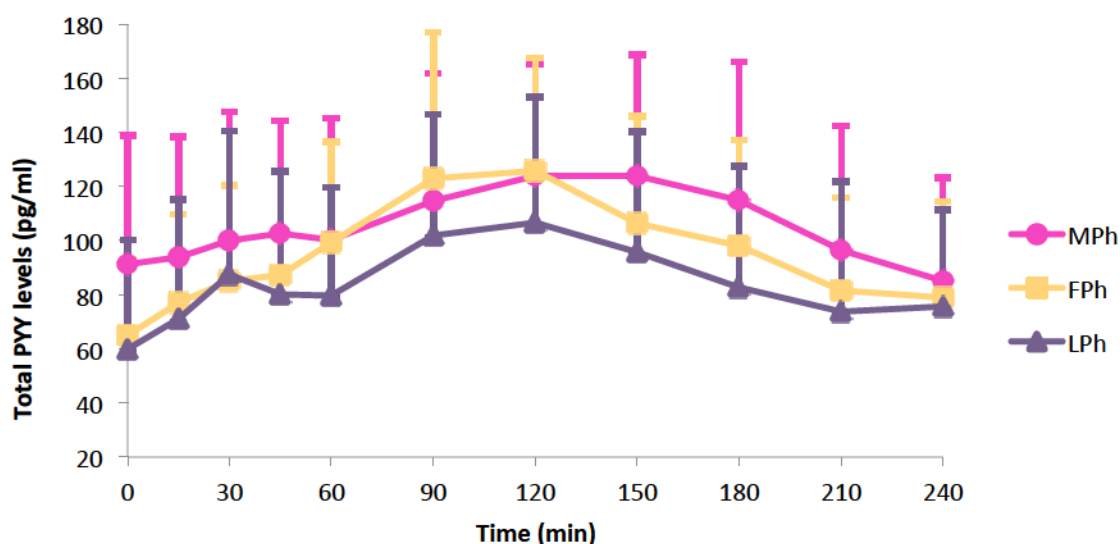


Fig 5.5. PYY levels at each time point in the different phases of the MC (Means \pm SD).

When looking at the change in PYY levels at every time point, we found that only time had a significant effect ($p < 0.001$), whereas phase made no statistical difference ($p = 0.846$) and there was not a phase \times time interaction effect ($p = 0.213$) (Fig 5.6).

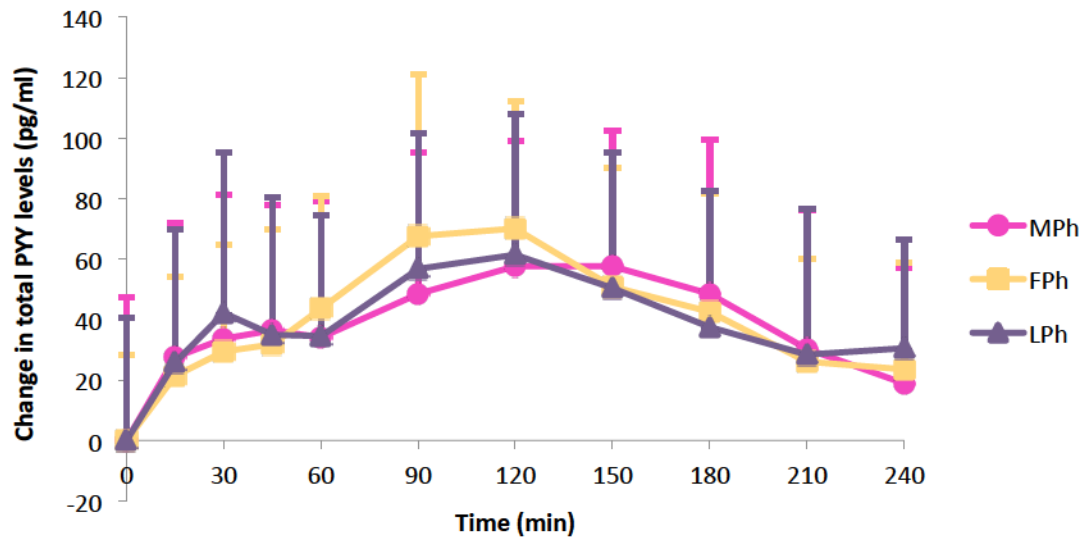


Fig 5.6. Change in PYY levels from baseline to each time point in the different phases of the MC (Means \pm SD).

There was a significant overall effect on PYY AUC at $t = 60, 120, 180$ and 240 , but only at $t = 60$, there was a significant difference between specific phases, i.e. LPh vs. MPh (4627 ± 2712 vs. 5883 ± 2601 pg/ml/min) ($p = 0.021$) (Fig 5.7). However the effects observed seem to mainly reflect that the PYY AUCs at $t = 120, 180$ and 240 were smaller in the LPh compared to the MPh ($p = 0.066, 0.129$ and 0.113 , respectively).

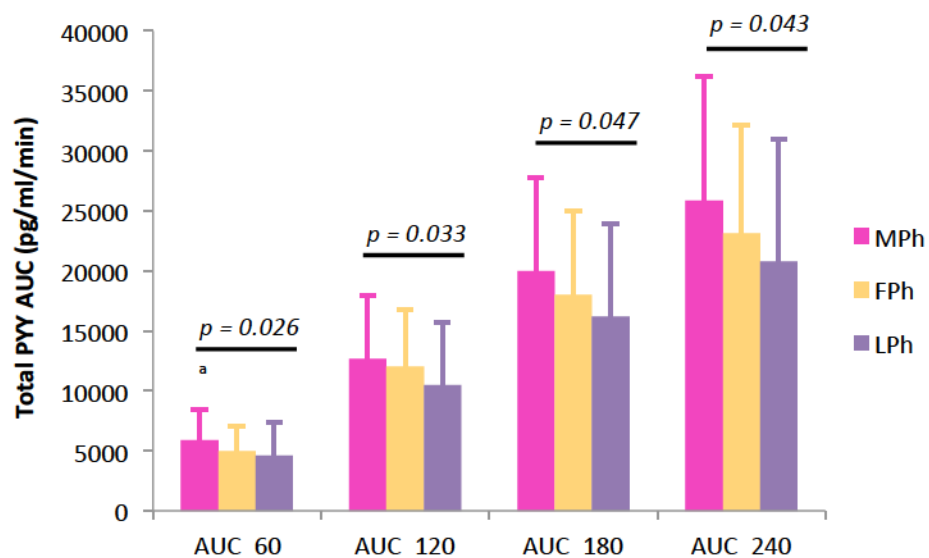


Fig 5.7. Total PYY AUCs at $t = 60, 120, 180$ and 240 in the different phases of the MC (Means \pm SD). ^a Significantly different to the LPh within the same time AUC.

5.3.5. Satiety ratings

There were no significant differences in AUC for any of the four satiety questions when analysing them in a two-way-ANOVA (time x phase) with the baseline measurements as covariates. AUC for the questions “*how hungry do you feel*” and “*how strong is your desire to eat*” showed to be the highest in hunger and strongest in desire to eat for every hour as follows: LPh > FPh > MPh. In contrast, for questions of “*How full do you feel?*” and “*How much food do you think you can eat?*” there was not a consistent pattern between phases in each of the time AUCs (Fig 5.8).

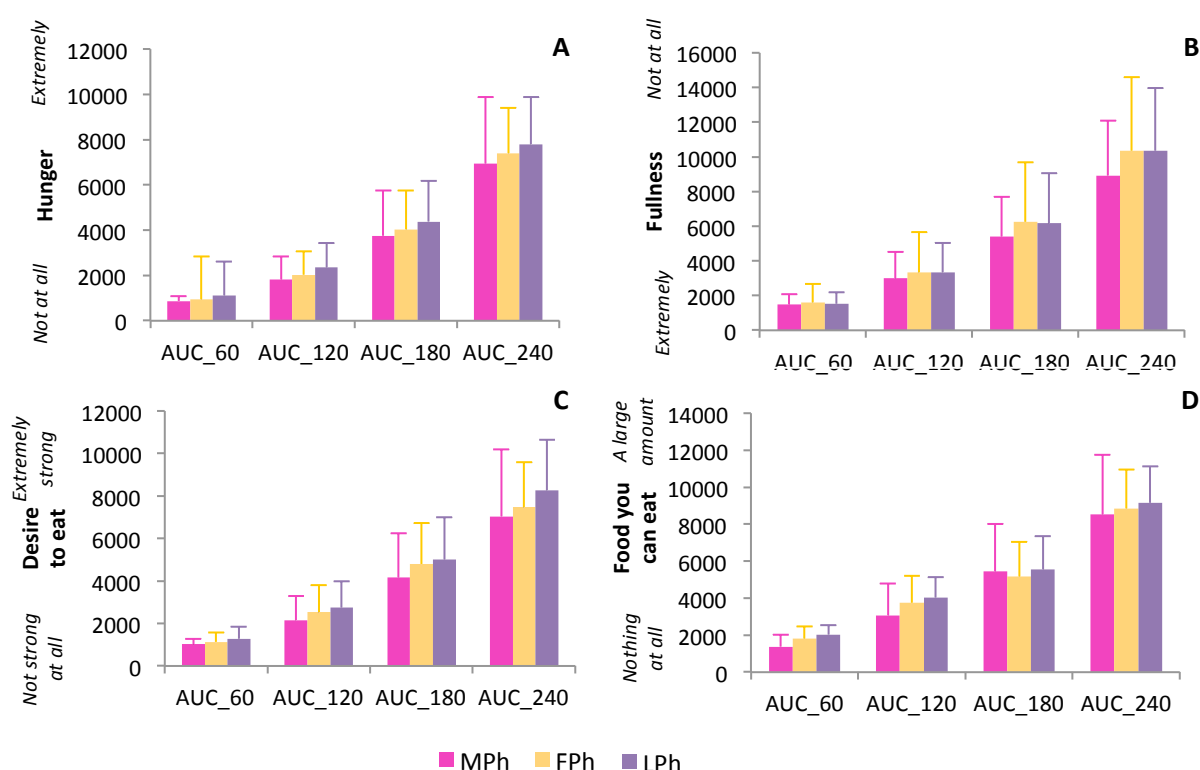


Fig 5.8. Total AUCs (mm/min) at t = 60, 120, 180 and 240 in the different phases of the MC for the four appetite sensations questions: (1) ‘How hungry do you feel?’ (A), (2) ‘How full do you feel?’ (B), (3) ‘How strong is your desire to eat?’ (C) and (4) ‘How much food do you think you can eat?’ (D). (Means ± SD)

5.3.6. Ad-libitum, post-lunch and averaged food intake

For this section of the results, a participant’s data was excluded as her eating behaviour and food diary analyses showed a strong indication that she was restricting her EI during the *ad-libitum* buffet-lunch as well as underreporting her food intake in the food diary. Specifically, when comparing her EE measured from the PA monitor against the EI from her food diary it

was found that her EI, as an average of the days measured, was 1807 kcal/d, whereas her EE was of 3045 kcal/d. That equals to 41 % difference. Considering that her predictive RMR would be of 1616 kcal/d (using Harris & Benedict formula), this amount of EI might not be sufficient to accomplish her energy requirements of the day. Moreover, the activity monitor and the life style questionnaire showed that she was a moderately active person, thus the data that was collected regarding her eating patterns did not seem genuine enough to be included.

During the buffet lunch there were no significant differences in EI, CHO, protein or fat intake between phases of the MC (Table 5.3). Similarly no differences were observed in food intake once participants left the laboratory. In addition, as an average of the three days in each MC phase, non-significant differences were found in food intake. Finally, food intake as an average of the day before and after the laboratory visit, i.e. food intake under free-living conditions did not change significantly for energy, CHO, fat or protein intake across the MC (Table 5.3).

Table 5.3. Food intake during and after the *ad libitum* lunch and as an average of the three measured days in each MC phase.

	MPh	FPh	LPh
<i>Ad libitum</i> lunch			
Energy (kcal)	931 ± 193	984 ± 178	956 ± 194
CHO (g)	113 ± 20	119 ± 20	116 ± 27
Fat (g)	38 ± 11	41 ± 10	39 ± 8
Protein (g)	29 ± 6	30 ± 6	30 ± 7
<i>After Ad libitum</i> lunch			
Energy (kcal)	1131 ± 339	1308 ± 660	1192 ± 485
CHO (g)	134 ± 47	156 ± 93	141 ± 54
Fat (g)	42 ± 14	52 ± 25	56 ± 31
Protein (g)	43 ± 21	39 ± 16	34 ± 18
Average of 3 days			
Energy (kcal)	2352 ± 358	2368 ± 604	2443 ± 412
CHO (g)	271 ± 41	274 ± 70	279 ± 54
Fat (g)	101 ± 21	97 ± 25	106 ± 22
Protein (g)	84 ± 17	77 ± 10	85 ± 15
Free-living conditions			
Energy (kcal)	2292 ± 146	2203 ± 598	2386 ± 520
CHO (g)	264 ± 52	255 ± 64	270 ± 67
Fat (g)	103 ± 26	91 ± 26	103 ± 27
Protein (g)	79 ± 19	70 ± 13	84 ± 20

Means ± SD

5.3.7. Relationships between PYY, GE, appetite feelings and EI

There was a significant moderate correlation between peak PYY and T_{half} and T_{asc} ($r = 0.396$ and 0.410 , $p = 0.041$ and 0.034). Moreover, there was a trend for a moderate correlation of PYY AUC at time 180 and 240 with T_{half} and T_{asc} ($r = 0.4$ for all, $p = 0.08$ and 0.07 for T_{half} and T_{asc} correlations, respectively).

AUCs from the VAS question 1 (*“how hungry do you feel”*) at $t = 240$ showed a significant moderate correlation with T_{asc} ($r = 0.416$, $p = 0.031$). No other correlations were found for appetite ratings with other GE parameters, PYY response or food intake.

No significant correlations were found between EI and PYY, GE or appetite feelings.

5.3.8. Relationships between appetite markers and the ovarian hormones

There was a moderate negative correlation between T_{half} and T_{asc} and P_4 levels ($r = -0.490$ and -0.426 , $p = 0.010$ and 0.027 , respectively). Moreover, T_{half} and T_{asc} were positively correlated to $E_2:P_4$ ratio ($r = 0.437$ and 0.407 , $p = 0.023$ and 0.035).

There were no correlations between PYY AUCs or peak PYY and the ovarian hormones.

Similarly, no correlations between appetite sensations or food intake and the ovarian hormones were found.

5.4. Discussion

The aim of this study was to investigate whether appetite responses vary after consuming the same breakfast in the different phases of the MC. This research is of importance in order to extend the current knowledge in appetite regulation in a subset of the adult population who seems to be at a higher risk of developing obesity than men (WHO 2015).

5.4.1. GE throughout the MC

Our results showed that the time to empty half of the breakfast from the stomach to the duodenum (T_{half}) was significantly different across the phases of MC, being on average 28 and

13 minutes quicker in the LPh compared to the FPh and MPh, respectively. Looking at the different GE parameters, it could be suggested that the reduction in the GE time (represented by T_{half}) was because of a significantly shorter T_{asc} and, potentially, a faster T_{lag} in the LPh compared to the other phases. This is, GE rate only became altered between phases after the initial delay in the rate of food emptying from the stomach, namely T_{lat} . Therefore, the effect seen in the GE time by the MC phase seems to respond to a change in the later stages of the GE process, when the highest GE rates are present (T_{asc}) and potentially, the time taken to the stomach to reach this stage (T_{lag}). Namely, in the LPh, high GE rates might have been reached quicker and once attained, these were sustained for a shorter period which resulted in a reduction of the time required to empty the same amount of food from the stomach when compared to the other two phases of the MC. Because T_{asc} was maintained for less time in the LPh, GE rates achieved during that period had to be of a higher velocity to achieve a shorter GE time.

Faster GE during the LPh in comparison to the FPh has previously been described (Brennan et al 2009), however, others have found opposite results (Gill et al 1987) or no differences (Horowitz et al 1985, Monés et al 1993) between these two phases, thus a definite position in this matter cannot be made with the available evidence. Discrepancies amongst studies might rely on different test meals in terms of calories and nutrient composition (Horowitz et al 1985) and the fact that some women might not have ovulated as that was not tested (Monés et al 1993).

Furthermore, attention is warranted as our outcome does not only support those who found differences between the LPh and the FPh (Brennan et al 2009), but also suggests that the GE effect seen in the LPh is large enough to be compared to the MPh, as well. As far as we know, this is the first study to add the MPh as another time point to investigate GE within the MC and our findings suggest that this should be included in future investigations.

5.4.2. PYY throughout the MC

To our knowledge, this is the first investigation to indicate that pre- and post-prandial PYY levels significantly change amongst the phases of the MC. The results of the study suggest that during the LPh there are lower PYY levels when participants are fasted, but also these have a smaller response when comparing it to the MPh. Moreover, this was kept consistently lower every post-prandial hour until the end of the test (4 hours later the start of the breakfast). The

MC effect on the PYY response seem to partly result from the significant differences found at baseline (when fasted) as the statistical significance was lost when looking at the change in PYY levels from baseline ($p = 0.213$).

PYY secretion is dependent on the presence of food in the lower intestine (ileum, colon) where the L-cells are located, thus by direct contact (Fu-Cheng et al 1995), but also by digestive events that occur at upper sections of the gastrointestinal tract, i.e. duodenum and stomach. Specifically, there is evidence that gastrin, which is known to stimulate the production of gastric acid, can inhibit the release of PYY as seen in rats (Gomez et al 1996). Meanwhile, the increase in gastric acid concentrations will trigger the synthesis of PYY as part of the ileal brake of the digestion process, thus creating a feedback loop between the upper and lower gastrointestinal tract. In some (Adamopoulos et al 1982) but not all (Frick et al 1990, Uvnäs-Msoberg et al 1989) studies, gastrin levels were elevated in the LPh when compared to the FPh which, provided this is the case of our participants, could partly explain the impairment in the PYY release and the consequent unavailability to reduce the GE time in the LPh. This was supported by the positive correlation found between T_{half} and PYY peak, and the tendency for a positive correlation with the PYY AUC at $t = 180$ and 240 .

Another potential mechanism that could have contributed to the different PYY responses would be changes in the CCK secretion. CCK release after the infusion of long-chain fatty acids in the duodenum has been shown to up-regulate PYY secretion by CCK-receptor 1 (Degen et al 2007), thus, if CCK secretion is inhibited in the LPh that could in turn impair PYY release. Although, Brennan et al (2009) found that CCK secretion was maintained across the MC, that could have been determined by the fact that participants only ingested a glucose drink and CHOs are known to be less effective in stimulating the CCK than fats (Hildebrand et al 1990), thus there could still be a potential for CCK modulating the changes in PYY secretion across the phases of the MC.

5.4.3. Ovarian hormones associations

One interesting finding of our study was the significant negative correlation between P_4 and T_{half} . Despite being only a moderate correlation, our results agree with Brennan et al's findings and corroborate the idea that the ovarian hormones might have an influence in GE. Furthermore, the fact that the ratio between E_2 and P_4 is also significantly correlated, suggests that both hormones are somehow related to the changes in the GE process.

Although our results did not indicate a direct association between PYY levels and the ovarian hormones, these may have exerted their influence by other factors involved in the digestive process e.g. GE, other appetite-hormonal secretions.

Considering the natural occurring changes in E_2 between the MPh and FPh it seemed necessary to investigate three rather than two phases and this was corroborated by the outcome of the study.

5.4.4. Food intake and appetite sensations throughout the MC

Although increases in food intake in the *ad-libitum* lunch were expected in the LPh as seen in previous literature (McNeil and Doucet 2012), our results did not find significant fluctuations in EI or macronutrient intake across the phases of the MC. In fact, even looking at food intake after the lunch buffet as well as the overall day, differences were negligible. This could be due to the fact that the majority of the food intake of the day (while in the laboratory) was already purposely kept constant, thus leaving little room for any changes. Nevertheless, food intake under the free-living conditions, which was 183 and 94 kcal/d higher in the LPh compared to the FPh and MPh, respectively, was not significantly different throughout the MC phases, either. Despite not reaching the statistical significance, fluctuations were within the spectrum of +50-100 kcal/d which are recognised to be of enough magnitude to induce the progressive development of obesity (Mozaffarian et al 2011).

The unchanged food intake during the lunch buffet, is somehow, expected since there were no significant differences in the appetite sensations post-breakfast, suggesting that food intake was responding to actual appetite perceptions and not to other extrinsic factors. However, direct correlations were not found between food intake and appetite sensations which manifests the difficulty in assessing subjective measurements. In fact, the only correlation found with appetite sensations, i.e. T_{asc} and hunger ratings, cannot be explained by the known literature. Since this correlation was only moderate ($r = 0.416$) and it appears to be opposite to what it would be expected, it could be suggested to respond to a random effect

Although, the assessment of food intake in a controlled setting presents important advantages, such as the availability to accurately quantify what it is consumed, it also presents several limitations that cannot be ignored. For instance, eating behaviour can be altered due to eating in a non-familiar and unnatural environment, or because of the expectations the participants believe that the researcher might have (Stubbs et al 1998). Nevertheless, we tried

to minimise this effect by providing a sensible variety of foods that the participant could be familiar with. On the other hand, although food diaries can avoid the limitations of the laboratory setting, they can also present different drawbacks such as the misreporting shown in one of our participants as well as in other studies (de Vries et al 1994). Therefore, both methods agreed on the idea that there were no significant alterations in food intake throughout the phases of the MC. Inconsistencies with other studies might rely on the limited sample size, although others have proved significant differences with the same number of participants (Dalvit-McPhillips 1983). Thus, differences between the latter study and ours could partly be due to the dietary assessment techniques employed i.e. dietary interview during 60 days vs. nine days of food diaries.

Finally, although PYY response significantly changed throughout the MC, the magnitude of the change (mean change in total PYY AUC at min 240 in LPh: 29 and 22% compared to the MPh and FPh, respectively) might not have been substantial enough to elicit modifications in appetite sensations and subsequent food intake.

5.4.5. Limitations

Despite GE time and PYY response showing to be significantly different across the MC, pairwise comparisons could not achieve the statistical significance and that could be due to the small sample size. In fact, if applied a t-test to compare T_{half} between the LPh and FPh or MPh, significant differences would have been found, however with the ANOVA and the Bonferroni correction, the statistical significance was diminished. Nevertheless, we employed the same size used by previous studies (Brennan et al 2009).

Our study did not distinguish the two different forms of PYY, i.e. PYY₁₋₃₆ and PYY₃₋₃₆. It is well known that with food intake PYY₁₋₃₆ is cleaved by the dipeptidyl peptidase IV to PYY₃₋₃₆ and that only the latter form has almost exclusive and high affinity to Y-2 receptors of the ARC. This is of relevance as Y-2 receptors are the only subtype of Y-receptors that can induce appetite and BW suppression by stimulating the activity of the α -MSH through the inhibition of the NPY release (Ballantyne 2006). Thus, although unlikely, changes in the concentrations of total PYY could respond to alterations in the proportion between the two forms of PYY. Thus it cannot be dismissed that lower PYY response in the LPh was mainly relying on a diminished PYY₁₋₃₆ secretion and conversion, therefore, inducing minimal changes in food intake. Future studies could improve our findings by measuring the two forms of PYY.

5.5. Conclusion

To our knowledge this is the first study to investigate GE time and PYY response after consuming the same breakfast three times in the MC in which ovarian hormones, E_2 and P_4 presented very distinguishable levels. Our results found significant differences in GE time and PYY response that suggest the LPh as the quickest in GE time with the smallest PYY response of the all MC phases. Appetite sensations or food intake were not affected by the MC. Finally changes in the GE time could be influenced by the fluctuations in the ovarian hormones.

Further research needs to be done to confirm the findings of our study and to have a better understanding of the underlying mechanisms for these changes in GE time and PYY response across the MC as they could potentially direct us to novel dieting strategies in women. Finally, our findings suggest that any functional food studies aimed to change satiation should take into account the likely modifications in the processing of food that women might experience throughout the MC by re-testing their products in the different MC phases to ultimately be able to demonstrate the effects of a dietary intervention in this population.

6. General discussion and future research

Sex differences in physiological factors involved in the regulation of energy homeostasis such as RMR, body composition and food intake have been studied for a very long time in animal models and humans (Arciero et al 1993, Asarian and Geary 2013, Lemieux et al 1993). All these factors are without doubt highly inter-related and dependent on each other, e.g. women tend to have a higher BF% than men which results in a smaller relative RMR that will ultimately reduce women's energy requirements in comparison to men. More importantly, these differences seem to be ultimately regulated by the sex steroid hormones i.e. oestrogens, progesterone and androgens. Since levels of E_2 and P_4 suffer cyclical fluctuations within a MC, it seems reasonable to question whether factors like the abovementioned are influenced by these hormones. Furthermore, the fact that the current global prevalence of overweight and obesity in adults of >18 years is greater in women than men might indicate that women are less efficient in achieving body energy balance (WHO 2015).

Therefore, this thesis was set up with the aim of extending the current knowledge in the regulation of energy metabolism in women within the MC. The areas of particular focus were the RMR and the appetite responses to a meal, therefore encompassing elements involved in both sides of the energy balance equation, i.e. EE and EI. Furthermore, we aimed to explore the role that E_2 and P_4 might have in the regulation of these and how both hormones are related with their respective salivary counterparts. Our key findings are summarised below:

- Saliva:plasma ratio for E_2 and P_4 changed significantly across the phases of the MC in NC women due to the more robust alterations in the concentrations of plasma vs. saliva.
- In NC women, only the LPh showed a significant and strong correlation between saliva and plasma P_4 . Correlations for E_2 were significant, but very weak.
- In HC users, both plasma ovarian hormones were suppressed whereas only salivary E_2 was reduced when compared to the NC group. Salivary P_4 suppression due to the intake of HC could not be confirmed due to the assay technique employed.
- Intra-subject variability in RMR was greater in NC compared to HC users.
- Increases in RMR in the LPh of NC women did not reach statistical significance despite showing clinically meaningful fluctuations, particularly in half of the participants studied.

- RMR in the LPh was significantly and negatively correlated to salivary, but not plasma, P_4 . This may explain some of the variance in an RMR predictive model, although BW might have confounded this association.
- There exist significant differences in GE time and PYY response to a standardized breakfast across the MC phases. These results show the LPh to be the quickest in GE time and with the smallest PYY response of the three MC phases. Furthermore, PYY response seemed to be influenced by the significant differences in fasting PYY levels.
- The significant correlations of T_{half} with P_4 and E_2-P_4 ratio suggest that both ovarian hormones are somehow related to the changes in the GE process throughout the MC.
- The introduction of the MPh as a different or an extra phase of the MC seems to be relevant when studying physiological changes across the MC, particularly in the GE and appetite hormones response.

The findings of our work cannot entirely prove that NC women exhibit cyclical changes in the regulation of the energy balance system within a MC, but they indicate that RMR can increase in the LPh of some NC women to levels that would ultimately affect their BW. Our participants did not exhibit any significant changes in their BW, therefore, either the increase in energy demands was compensated in the other phases of the MC by a relative lower RMR or changes in the other side of the energy equation were made to compensate the calorie deficit within the same phase. Indeed, our outcome also suggests that the consequent increase in TEE could be self-compensated by modifying the digestion process of certain foods. Specifically, by the joint action of a quicker GE with a smaller PYY response, women would experience a reduced satiation in the LPh that would result in an increase in food intake and thus the maintenance of BW. Although the changes in satiety feelings and food intake could not be shown by our results, a great number of studies in the past have demonstrated how EI increases in the LPh compared to the FPh. Thus, our findings support the idea that the regulatory mechanisms by which energy balance is achieved, might experience various alterations according to the phases of the MC. It would not be sensible to assume that the compensatory mechanisms studied in this thesis (i.e. GE time and PYY response) are the only two factors changing across the phases of the MC since as described in the literature review there are many factors regulating what, how much and when do we eat. For instance, there is evidence certain areas of the corticolimbic brain of women only respond to high calorie (but not low calorie) food cues in the LPh

which has been related to the changes in food preferences within the MC (Van Vugt 2010). Whether any of these regulatory changes are beneficial or not in the maintenance of BW in the long term remains unknown, although a priori it seems that women should be as efficient as men, yet men's energy regulatory systems appear to be more stable.

Reasons behind the global tendency of higher obesity prevalence in women than men may more likely rely on a combination of external and internal factors (e.g. socioeconomic status, PA levels) rather than a unique sex-related cause. Nevertheless, taking into account the outcome of our investigations, it could be questioned whether pre-menopausal women might be more or less prone to imbalances in energy balance with an impact on their BW and composition due to alterations in their homeostatic system within a MC.

If the findings of our research could be confirmed by further investigations and indeed energy regulation varied according to the phase of the MC, nutritional strategies for the prevention of obesity in the female population could follow a new approach. For example, GP's/nutritionists could provide information to women with the aim to make them aware that if they feel hungrier or have a higher food consumption pre-menstrually, they should bear in mind that this is to be expected and that they could increase their intake but they should also use sources of food known to be healthy and not surrender to the high caloric cravings.

Despite being unable to assess the direct underlying mechanisms of the alterations in RMR and GE and PYY response within a MC, it could be suggested that the fluctuations in the ovarian hormones have an influence by affecting the homeostatic contributors studied in the present investigation and others, e.g. leptin, insulin (Asarian and Geary 2013, Shi et al 2009). In addition, the fact that only salivary (but not plasma) P_4 had a significant correlation with RMR might support the idea that salivary ovarian hormones act differently or rather, have a different association with some of the metabolic parameters to the plasma counterparts.

In summary, the novelty of our work relies on the different assessment method employed for the RMR (Study 2) which provides a more accurate output of the changes in RMR within a MC. Furthermore, by adding the measurement of the salivary ovarian hormones we discovered the influence of salivary P_4 on RMR as it contributed in the variance of RMR and it was significantly and negatively correlated to it. Finally, as far as we know, this is the first

investigation (Study 3) to explore and find that PYY response to a meal changes significantly throughout the MC and that this was related to differences shown in GE.

6.1. Limitations, implications, and future research

The main limitation presented throughout our work is the restriction in number of participants due to the constraints of the methodology employed (for example, invasiveness, time invested, inclusion criteria) and time available (i.e. from the time of consent until the completion of a participant, at least one month is required). Nevertheless, this is the nature of research in this area and the sample size effect was reduced by using the same participants in the different phases of the MC.

The findings of our investigation suggest that nutrition practitioners and researchers should bear in mind that their female clients / participants might experience fluctuations in their energy demands and might process differently the food consumed depending on the phases of the MC. Therefore, MC phase should not be ignored and researchers should aim to control it, but also investigate it. Furthermore, the collection of salivary ovarian hormones should be encouraged in future research as this not only presents obvious benefits related to their collection technique, but also it can offer a different and, possibly, more physiologically meaningful information about the ovarian hormone concentrations.

To conclude, future areas of research could include the following:

- Further investigations on formulating EIA to measure salivary P_4 in HC users as the one employed in our project seemed to cross-react with the HC.
- The study of salivary and plasma ovarian hormones along with the measurement of their main blood carriers, i.e. SHBG and CBG, in NC and HC users.
- The association with high or low-CV in RMR with long term BW maintenance.
- The investigation of other meal-appetite regulators (e.g. CCK) as possible contributors in the changes in the digestive process within the MC.
- The study of appetite responses and food intake in women who are using HC.
- The exploration of the parameters researched in our work but in a population of obese pre-menopausal women. Our studies have been based on samples of normal-overweight women and since states of obesity can affect the reproductive

system it would not be sensible to assume that our findings will be transferable in obese women.

- Similarly, women with polycystic ovary syndrome (PCOS) i.e. women who suffer an imbalance in their sex hormone concentrations, would be a population of great interest to study since different results to the ones observed in the present work would be expected due to their inability to ovulate and their altered levels in sex hormones.
- Further conditions, such as women suffering from eating disorders would be another interesting population to study. Women with anorexia nervosa usually display amenorrhea (i.e. irregular or absent menstrual period) which is linked to the inhibition of the HPG axis as a consequence of the starvation state their bodies are exposed to. Furthermore, as previously exposed in the literature review, appetite regulators such as leptin, ghrelin and GLP-1 are affected by this disease, therefore it would be interesting to study whether responses to the same meal are kept constant in women who still have their MC but may display amenorrhea.

References

- Adamopoulos DA, Dessypris A, Xanthopoulos J and Chryssicopoulos E (1982) Gastrin secretion in the menstrual cycle and pregnancy. *Hepato-gastroenterology* 29(1): 24–6.
- Adekunle AO, Kim JB, Collins WP and Whitehead MI (1989) Progesterone in saliva as an index of ovarian function. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 28(1): 45–51.
- Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM and Bloom SR (1985) Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89(5): 1070–1077.
- Ahn RS, Choi JH, Choi BC, Kim JH, Lee SH and Sung SS (2011) Cortisol, estradiol-17 β , and progesterone secretion within the first hour after awakening in women with regular menstrual cycles. *The Journal of endocrinology* 211(3): 285–95.
- Allen SS, Hatsukami D, Christianson D and Brown S (2000) Energy intake and energy expenditure during the menstrual cycle in short-term smoking cessation. *Addictive behaviors* 25(4): 559–72.
- Arciero PJ, Goran MI and Poehlman ET (1993) Resting metabolic rate is lower in women than in men. *Journal of applied physiology* 75(6): 2514–20.
- Asarian L and Geary N (2013) Sex differences in the physiology of eating. *American journal of physiology. Regulatory, integrative and comparative physiology* 305(11): R1215–67.
- Baecke JA, Burema J and Frijters JE (1982) A short questionnaire for the measurement of habitual physical activity in epidemiological studies. *The American journal of clinical nutrition* 36(5): 936–42.
- Ballantyne GH (2006) Peptide YY(1-36) and peptide YY(3-36): Part II. Changes after gastrointestinal surgery and bariatric surgery. *Obesity surgery* 16(6): 795–803.
- Bao A-M, Liu R-Y, van Someren EJW, Hofman M a, Cao Y-X and Zhou J-N (2003) Diurnal rhythm of free estradiol during the menstrual cycle. *European journal of endocrinology / European Federation of Endocrine Societies* 148(2): 227–32.
- Barr S, Janelle K and Prior J (1995) Energy intakes are higher during the luteal phase of ovulatory menstrual cycles. *Am J Clin Nutr* 61(1): 39–43.
- Barton M and Wiesner B (1945) Thermogenic effect of progesterone. *The Lancet* 246(6378): 671–672.
- Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghattei MA and Bloom SR (2003) Inhibition of food intake in obese subjects by peptide YY3-36. *The New England journal of medicine* 349(10): 941–8.
- Bellem A, Meiyappan S, Romans S and Einstein G (2011) Measuring estrogens and progestagens in humans: an overview of methods. *Gender medicine* 8(5): 283–99.
- Bentley PJ (1980) *Endocrine Pharmacology: Physiological Basis and Therapeutic Applications*. CUP Archive.
- Bisdee JT, James WPT and Shaw MA (1989) Changes in energy expenditure during the

menstrual cycle. *British Journal of Nutrition* 61(02): 187.

Bloom B, Chaikoff IL and Reinhardt WO (1951) Intestinal Lymph as Pathway for Transport of Absorbed Fatty Acids of Different Chain Lengths. *Am J Physiol -- Legacy Content* 166(2): 451–455.

Body Media (2006) *SenseWear body monitoring system. Instructions For Use*. Pittsburgh, USA.

De Boever J, Kohen F and Vandekerckhove D (1986) DirectSolid-PhaseChemiluminescence Immunoassay for Salivary Progesterone. 32(5): 763–767.

Bolaji II (1994) Sero-salivary progesterone correlation. *International Journal of Gynecology and Obstetrics* 45(2): 125–131.

Bosy-Westphal A, Müller MJ, Boschmann M, Klaus S, Kreymann G, Lührmann PM, Neuhäuser-Berthold M, Noack R, Pirke KM, Platte P, Selberg O and Steiniger J (2008) Grade of adiposity affects the impact of fat mass on resting energy expenditure in women. *British Journal of Nutrition* 101(04): 466.

Bourque J, Sulon J, Demey-Ponsart E, Sodoyez JC and Gaspard U (1986) A simple, direct radioimmunoassay for salivary progesterone determination during the menstrual cycle. *Clinical Chemistry* 32(6): 948–951.

Brennan IM, Feltrin KL, Nair NS, Hausken T, Little TJ, Gentilcore D, Wishart JM, Jones KL, Horowitz M and Feinle-Bisset C (2009) Effects of the phases of the menstrual cycle on gastric emptying, glycemia, plasma GLP-1 and insulin, and energy intake in healthy lean women. *American journal of physiology. Gastrointestinal and liver physiology* 297(3): G602–10.

Bryant M, Truesdale KP and Dye L (2006) Modest changes in dietary intake across the menstrual cycle: implications for food intake research. *British Journal of Nutrition* 96(05).

Buffenstein R, Poppitt SD, McDevitt RM and Prentice AM (1995) Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. *Physiology & behavior* 58(6): 1067–1077.

Cedard L, Janssens Y, Tanguy G and Zorn JR (1984) Radioimmunoassay of plasma and salivary progesterone during the menstrual cycle. *Journal of Steroid Biochemistry* 20(1): 487–490.

Chung S-C, Bond EF and Jarrett ME (2010) Food intake changes across the menstrual cycle in Taiwanese women. *Biological research for nursing* 12(1): 37–46.

Clearblue (2013) *Clear Blue Advanced Fertility Monitor*. Geneva, Switzerland.

Clegg M and Shafat A (2010) Energy and macronutrient composition of breakfast affect gastric emptying of lunch and subsequent food intake, satiety and satiation. *Appetite*. Elsevier Ltd 54(3): 517–23.

Coll AP, Farooqi IS and O’Rahilly S (2007) The hormonal control of food intake. *Cell* 129(2): 251–62.

Compher C, Frankenfield D, Keim N and Roth-Yousey L (2006) Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *Journal of*

the American Dietetic Association 106(6): 881–903.

Crawley JN and Corwin RL (1994) Biological actions of cholecystokinin. *Peptides* 15(4): 731–755.

Curtis V, Henry C, Birch E and Ghusain-Choueiri A (1996) Intraindividual variation in the basal metabolic rate of women: Effect of the menstrual cycle. *American Journal of Human Biology* 8(5): 631–639.

Curtis V, Henry C and Ghusain-Choueiri A (1996) Basal metabolic rate of women on the contraceptive pill. *European journal of clinical nutrition* 50(5): 319–22.

Dafopoulos K, Sourlas D, Kallitsaris A, Pournaras S and Messinis IE (2009) Blood ghrelin, resistin, and adiponectin concentrations during the normal menstrual cycle. *Fertility and sterility*. Elsevier Ltd 92(4): 1389–94.

Dalvit-McPhillips SP (1983) The effect of the human menstrual cycle on nutrient intake. *Physiology & behavior* 31(2): 209–12.

Day DS, Gozansky WS, Van Pelt RE, Schwartz RS and Kohrt WM (2005) Sex hormone suppression reduces resting energy expenditure and {beta}-adrenergic support of resting energy expenditure. *The Journal of clinical endocrinology and metabolism* 90(6): 3312–7.

Deane AM, Nguyen NQ, Stevens JE, Fraser RJL, Holloway RH, Besanko LK, Burgstad C, Jones KL, Chapman MJ, Rayner CK and Horowitz M (2010) Endogenous glucagon-like peptide-1 slows gastric emptying in healthy subjects, attenuating postprandial glycemia. *The Journal of clinical endocrinology and metabolism* 95(1): 215–21.

Degen L, Drewe J, Piccoli F, Gräni K, Oesch S, Bunea R, D'Amato M and Beglinger C (2007) Effect of CCK-1 receptor blockade on ghrelin and PYY secretion in men. *American journal of physiology. Regulatory, integrative and comparative physiology* 292(4): R1391–9.

Delhanty PJD, Neggers SJ and van der Lely AJ (2012) Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. *European journal of endocrinology* 167(5): 601–8.

Diffey B, Piers LS, Soares MJ and O'dea K (1997) The effect of oral contraceptive agents on the basal metabolic rate of young women. *British Journal of Nutrition* 77(06): 853.

Dye L and Blundell JE (1997) Menstrual cycle and appetite control: implications for weight regulation. *Human reproduction (Oxford, England)* 12(6): 1142–51.

EFSA (2013) Scientific Opinion on Dietary Reference Values for energy. *EFSA Journal* 11(1): 1–112.

Elliott KJ, Cable NT, Reilly T and Diver MJ (2003) Effect of menstrual cycle phase on the concentration of bioavailable 17-beta oestradiol and testosterone and muscle strength. *Clinical science (London, England : 1979)* 105(6): 663–9.

EMD Millipore Corporation (2014) *ELISA kit Human Leptin Cat. # ESHL-80SK*. USA.

Endrikat J, Blode H, Gerlinger C, Rosenbaum P and Kuhn W (2002) A pharmacokinetic study with a low-dose oral contraceptive containing 20 microg ethinylestradiol plus 100 microg levonorgestrel. *The European journal of contraception & reproductive health care : the official journal of the European Society of Contraception* 7(2): 79–90.

- Evans JJ (1986) Progesterone in saliva does not parallel unbound progesterone in plasma. *Clinical chemistry* 32(3): 542–4.
- Farooqi I, Keogh J, Kamath S, Jones S, Gibson W, Trussell R, Jebb S, Lip G and O’Rahilly S (2001) Partial leptin deficiency and human adiposity. *Nature*. Nature Publishing Group 414(6859): 34–5.
- Farooqi IS and O’Rahilly S (2009) Leptin: a pivotal regulator of human energy homeostasis. *The American journal of clinical nutrition* 89(3): 980S–984S.
- Feher J (2012) *Quantitative Human Physiology: An Introduction* (1st edition). Academic Press.
- Fehring RJ, Schneider M and Raviele K (2006) Variability in the phases of the menstrual cycle. *Journal of obstetric, gynecologic, and neonatal nursing : JOGNN / NAACOG* 35(3): 376–84.
- Ferin M, Jewelewicz R and Warren M (1993) *The menstrual cycle. Physiology, Reproductive Disorders, and Infertility*. New York: Oxford University Press.
- Forman J, Miller W, Szymanski L and Fernhall B (1998) Differences in resting metabolic rates of inactive obese African-American and Caucasian women. *International journal of obesity and related metabolic disorders* 22(3): 215–21.
- FPA (2007) *Contraception: patterns of use factsheet*. . Available at: <http://www.fpa.org.uk/factsheets/contraception-patterns-use> (accessed 06/11/15).
- Frank TC, Kim GL, Krzemien A and Van Vugt DA (2010) Effect of menstrual cycle phase on corticolimbic brain activation by visual food cues. *Brain research*. Elsevier B.V. 1363: 81–92.
- Frankenfield DC, Rowe WA, Smith JS and Cooney RN (2003) Validation of several established equations for resting metabolic rate in obese and nonobese people. *Journal of the American Dietetic Association* 103(9): 1152–9.
- Frayn KN (1983) Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55(2): 628–634.
- Frick G, Bremme K, Sjögren C, Lindén A and Uvnäs-Moberg K (1990) Plasma levels of cholecystokinin and gastrin during the menstrual cycle and pregnancy. *Acta obstetrica et gynecologica Scandinavica* 69(4): 317–20.
- Frye C (2006) An overview of oral contraceptives: mechanism of action and clinical use. *Neurology* 66(6 Suppl 3): S29–36.
- Fu-Cheng X, Anini Y, Chariot J, Voisin T, Galmiche JP and Rozé C (1995) Peptide YY release after intraduodenal, intraileal, and intracolonic administration of nutrients in rats. *Pflügers Archiv : European journal of physiology* 431(1): 66–75.
- Germain N, Galusca B, Le Roux CW, Bossu C, Ghattei MA, Lang F, Bloom SR and Estour B (2007) Constitutional thinness and lean anorexia nervosa display opposite concentrations of peptide YY, glucagon-like peptide 1, ghrelin, and leptin. *The American journal of clinical nutrition* 85(4): 967–71.
- Ghoos YF, Maes BD, Geypens BJ, Mys G, Hiele MI, Rutgeerts PJ and Vantrappen G (1993) Measurement of gastric emptying rate of solids by means of a carbon-labeled octanoic acid

breath test. *Gastroenterology* 104(6): 1640–7.

Gil Y, Fagundes R, Santos E, Calvo M and Bernardine J (2009) Relation of menstrual cycle and alimentary consumption of women. *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*. Elsevier Ltd 4(5): e257–e260.

Gill RC, Murphy PD, Hooper HR, Bowes KL and Kingma YJ (1987) Effect of the menstrual cycle on gastric emptying. *Digestion* 36(3): 168–74.

Goedecke JH, St Clair Gibson A, Grobler L, Collins M, Noakes TD and Lambert E V (2000) Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes. *American journal of physiology. Endocrinology and metabolism* 279(6): E1325–34.

Goldzieher JW and Stanczyk FZ (2008) Oral contraceptives and individual variability of circulating levels of ethinyl estradiol and progestins. *Contraception* 78(1): 4–9.

Gomez G, Padilla L, Udupi V, Tarasova N, Sundler F, Townsend CM, Thompson JC and Greeley GH (1996) Regulation of peptide YY homeostasis by gastric acid and gastrin. *Endocrinology* 137(4): 1365–9.

Gortari P De and Joseph-Bravo P (2006) Neuroendocrine regulation of energy homeostasis. *Molecular endocrinology* 661(2): 65–85.

Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE and Reeve JR (1994) Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regulatory peptides* 51(2): 151–9.

Granger DA, Hibel LC, Fortunato CK and Kapelewski CH (2009) Medication effects on salivary cortisol: tactics and strategy to minimize impact in behavioral and developmental science. *Psychoneuroendocrinology* 34(10): 1437–48.

Gröschl M (2008) Current status of salivary hormone analysis. *Clinical chemistry* 54(11): 1759–69.

Guida M, Tommaselli GA, Palomba S, Pellicano M, Moccia G, Di Carlo C and Nappi C (1999) Efficacy of methods for determining ovulation in a natural family planning program. *Fertility and sterility* 72(5): 900–4.

Guo Y, Ma L, Enriori PJ, Koska J, Franks PW, Brookshire T, Cowley MA, Salbe AD, Delparigi A and Tataranni PA (2006) Physiological evidence for the involvement of peptide YY in the regulation of energy homeostasis in humans. *Obesity (Silver Spring, Md.)* 14(9): 1562–70.

Hameed S, Dhillon WS and Bloom SR (2009) Gut hormones and appetite control. *Oral diseases* 15(1): 18–26.

Hampson E and Young EA (2007) *Sex Differences in the Brain : From Genes to Behavior: From Genes to Behavior*. USA: Oxford University Press.

Haycock GB, Schwartz GJ and Wisotsky DH (1978) Geometric method for measuring body surface area: a height-weight formula validated in infants, children, and adults. *The Journal of pediatrics* 93(1): 62–6.

Heffner LJ and Schust DJ (2014) *Reproductive System at a Glance*. Somerset: Wiley.

Henry CJK, Hayter J and Rees DG (1989) The constancy of basal metabolic rate in free-living

male subjects. *European journal of clinical nutrition* 43(10): 727–31.

Henry CJK, Lightowler HJ and Marchini J (2003) Intra-individual variation in resting metabolic rate during the menstrual cycle. *The British journal of nutrition* 89(6): 811–7.

Hildebrand P, Beglinger C, Gyr K, Jansen JB, Rovati LC, Zuercher M, Lamers CB, Setnikar I and Stalder GA (1990) Effects of a cholecystokin receptor antagonist on intestinal phase of pancreatic and biliary responses in man. *The Journal of clinical investigation* 85(3): 640–6.

Hill BR, De Souza MJ and Williams NI (2011) Characterization of the diurnal rhythm of peptide YY and its association with energy balance parameters in normal-weight premenopausal women. *American journal of physiology. Endocrinology and metabolism* 301(2): E409–15.

Hill JO, Wyatt HR, Reed GW and Peters JC (2003) Obesity and the environment: where do we go from here? *Science (New York, N.Y.)* 299(5608): 853–5.

Hirschberg AL (2012) Sex hormones, appetite and eating behaviour in women. *Maturitas*. Elsevier Ireland Ltd 71(3): 248–56.

Hofman LF (2001) Human saliva as a diagnostic specimen. *The Journal of nutrition* 131(5): 1621S–5S.

Horowitz M, Maddern GJ, Chatterton BE, Collins PJ, Petrucco OM, Seamark R and Shearman DJ (1985) The normal menstrual cycle has no effect on gastric emptying. *British journal of obstetrics and gynaecology* 92(7): 743–6.

Horton TJ, Miller EK, Glueck D and Tench K (2002) No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. *American journal of physiology. Endocrinology and metabolism* 282(4): E752–62.

Howards PP, Schisterman EF, Wactawski-Wende J, Reschke JE, Frazer AA and Hovey KM (2009) Timing clinic visits to phases of the menstrual cycle by using a fertility monitor: the BioCycle Study. *American journal of epidemiology* 169(1): 105–12.

Howe JC, Rumpler W V and Seale JL (1993) Energy expenditure by indirect calorimetry in premenopausal women: variation within one menstrual cycle. *The Journal of Nutritional Biochemistry* 4(5): 268–273.

Hu J, Zhang Z, Shen W-J and Azhar S (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutrition & metabolism* 7(1): 47.

Jankowski CM, Ben-Ezra V, Gozansky WS and Scheaffer SE (2004) Effects of oral contraceptives on glucoregulatory responses to exercise. *Metabolism* 53(3): 348–352.

Javed F, He Q, Davidson LE, Thornton JC, Albu J, Boxt L, Krasnow N, Elia M, Kang P, Heshka S and Gallagher D (2010) Brain and high metabolic rate organ mass: contributions to resting energy expenditure beyond fat-free mass. *The American journal of clinical nutrition* 91(4): 907–12.

Johnson RK (2002) Dietary intake--how do we measure what people are really eating? *Obesity research* 10 Suppl 1: 63S–68S.

- Johnson WG, Corrigan SA, Lemmon CR, Bergeron KB and Crusco AH (1994) Energy regulation over the menstrual cycle. *Physiology & behavior* 56(3): 523–7.
- Karl JP, Young AJ, Rood JC and Montain SJ (2013) Independent and combined effects of eating rate and energy density on energy intake, appetite, and gut hormones. *Obesity (Silver Spring, Md.)* 21(3): E244–52.
- Kennedy S, Ryan L, Fraser A and Clegg ME (2014) Comparison of the GEM and the ECAL indirect calorimeters against the Deltatrac for measures of RMR and diet-induced thermogenesis. *Journal of Nutritional Science*. Cambridge University Press 3: e52.
- Kimm SY, Glynn NW, Aston CE, Poehlman ET and Daniels SR (2001) Effects of race, cigarette smoking, and use of contraceptive medications on resting energy expenditure in young women. *American journal of epidemiology* 154(8): 718–24.
- Kissileff HR, Thornton JC, Torres MI, Pavlovich K, Mayer LS, Kalari V, Leibel RL and Rosenbaum M (2012) Leptin reverses declines in satiation in weight-reduced obese humans. *The American journal of clinical nutrition* 95(2): 309–17.
- Kjems LL, Holst JJ, Vølund A and Madsbad S (2003) The Influence of GLP-1 on Glucose-Stimulated Insulin Secretion. *Diabetes* 52(April 2002): 380–386.
- Kolaczynski J, Ohannesian J, Considine R, Marco C and Caro J (1996) Response of leptin to short-term and prolonged overfeeding in humans. *The Journal of clinical endocrinology and metabolism* 81(11): 4162–5.
- Konishi S, Brindle E, Guyton A and O'Connor KA (2012) Salivary concentration of progesterone and cortisol significantly differs across individuals after correcting for blood hormone values. *American journal of physical anthropology* 149(2): 231–41.
- Kruger J, Galuska DA, Serdula MK and Jones DA (2004) Attempting to lose weight: specific practices among U.S. adults. *American journal of preventive medicine* 26(5): 402–6.
- Lee KA (1988) Circadian Temperature Rhythms in Relation to Menstrual Cycle Phase. *Journal of Biological Rhythms* 3(3): 255–263.
- Lemieux S, Prud'homme D, Bouchard C, Tremblay A and Després JP (1993) Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. *The American journal of clinical nutrition* 58(4): 463–7.
- Li ET, Tsang LB and Lui SS (1999) Menstrual cycle and voluntary food intake in young Chinese women. *Appetite* 33(1): 109–18.
- Liao PV and Dollin J (2012) Half a century of the oral contraceptive pill. *Canadian Family Physician* 58: 757–760.
- Liddle RA, Goldfine ID, Rosen MS, Taplitz RA and Williams JA (1985) Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *The Journal of clinical investigation* 75(4): 1144–52.
- Liening SH, Stanton SJ, Saini EK and Schultheiss OC (2010) Salivary testosterone, cortisol, and progesterone: two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels. *Physiology & behavior*. Elsevier Inc. 99(1): 8–16.

- Livingstone MBE and Black AE (2003) Markers of the validity of reported energy intake. *The Journal of nutrition* 133 Suppl : 895S–920S.
- Lopez LM, Edelman A, Chen-Mok M, Trussell J and Helmerhorst FM (2011) Progestin-only contraceptives: effects on weight. *The Cochrane database of systematic reviews* (4): CD008815.
- Lu YC, Bentley GR, Gann PH, Hodges KR and Chatterton RT (1999) Salivary estradiol and progesterone levels in conception and nonconception cycles in women: Evaluation of a new assay for salivary estradiol. *Fertility and Sterility* 71(5): 863–868.
- Lundberg K, Hilke S, Nordin C, Theodorsson E and Josefsson A (2010) Cholecystokinin in plasma and cerebrospinal fluid--a study in healthy young women. *Peptides* 31(8): 1625–1628.
- Lynch NA, Ryan AS, Berman DM, Sorkin JD and Nicklas BJ (2002) Comparison of VO₂max and disease risk factors between perimenopausal and postmenopausal women. *Menopause* 9(6): 456–62.
- Lyons PM, Truswell AS, Mira M, Vizzard J and Abraham SF (1989) Reduction of food intake in the ovulatory phase of the menstrual cycle. *The American journal of clinical nutrition* 49(6): 1164–8.
- Malagelada J-R and Azpiroz F (2010) *Determinants of gastric emptying and transit in the small intestine*. Hoboken, NJ, USA: John Wiley & Sons, Inc.
- Mantzoros CS, Magkos F, Brinkoetter M, Sienkiewicz E, Dardeno TA, Kim S-Y, Hamnvik O-PR and Koniaris A (2011) Leptin in human physiology and pathophysiology. *American journal of physiology. Endocrinology and metabolism* 301(4): E567–84.
- Margetic S, Gazzola C, Pegg GG and Hill RA (2002) Leptin: a review of its peripheral actions and interactions. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 26(11): 1407–33.
- Matsuo T, Saitoh S and Suzuki M (1998) Resting metabolic rate and diet-induced thermogenesis during each phase of the menstrual cycle in healthy young women. *Journal of clinical biochemistry and nutrition* 25(2): 97–107.
- Matsuo T, Saitoh S and Suzuki M (1999) Effects of the menstrual cycle on excess postexercise oxygen consumption in healthy young women. *Metabolism* 48(3): 275–277.
- Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD and Urban RJ (1998) Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *The Journal of clinical endocrinology and metabolism* 83(6): 1886–92.
- Mauvais-Jarvis F, Clegg DJ and Hevener AL (2013) The role of estrogens in control of energy balance and glucose homeostasis. *Endocrine reviews* 34(3): 309–38.
- McNeil J and Doucet É (2012) Possible factors for altered energy balance across the menstrual cycle: a closer look at the severity of PMS, reward driven behaviors and leptin variations. *European journal of obstetrics, gynecology, and reproductive biology* 163(1): 5–10.
- McNeill G, Bruce AC, Ralph A and James WP (1988) Inter-individual differences in fasting

nutrient oxidation and the influence of diet composition. *International journal of obesity* 12(5): 455–63.

Meijer G and Westerterp K (1992) Sleeping metabolic rate in relation to body composition and the menstrual cycle. *The American journal of clinical nutrition*.

Melzer K (2011) Carbohydrate and fat utilization during rest and physical activity. *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*. Elsevier 6(2): e45–e52.

Messini CI, Dafopoulos K, Malandri M, Georgoulas P, Anifandis G and Messinis IE (2013) Growth hormone response to submaximal doses of ghrelin remains unchanged during the follicular phase of the cycle. *Reproductive biology and endocrinology* 11: 36.

Moghissi KS (1976) Accuracy of basal body temperature for ovulation detection. *Fertility and sterility* 27(12): 1415–21.

Monés J, Carrió I, Calabuig R, Estorch M, Sainz S, Berná L and Vilardell F (1993) Influence of the menstrual cycle and of menopause on the gastric emptying rate of solids in female volunteers. *European Journal of Nuclear Medicine* 20(7).

Monga AK and Dobbs SP (2011) *Gynaecology by ten teachers by Monga, Ash K, Dobbs, Stephen P* (19th edition). Taylor & Francis Group.

Moschos S, Chan J and Mantzoros C (2002) Leptin and reproduction: a review. *Fertility and sterility* 77(3).

Mozaffarian D, Hao T, Rimm EB, Willett WC and Hu FB (2011) Changes in diet and lifestyle and long-term weight gain in women and men. *The New England journal of medicine* 364(25): 2392–404.

Näslund E and Hellström PM (2013) *Satiation, Satiety and the Control of Food Intake. Satiation, Satiety and the Control of Food Intake*. Elsevier.

Natalucci G, Riedl S, Gleiss A, Zidek T and Frisch H (2005) Spontaneous 24-h ghrelin secretion pattern in fasting subjects: maintenance of a meal-related pattern. *European journal of endocrinology* 152(6): 845–50.

Nelson KM, Weinsier RL, Long CL and Schutz Y (1992) Prediction of resting energy expenditure from fat-free mass and fat mass. *The American journal of clinical nutrition* 56(5): 848–856.

Nieuwenhuizen AG, Hochstenbach-Waelen A, Veldhorst MAB, Westerterp KR, Engelen MPKJ, Brummer R-JM, Deutz NEP and Westerterp-Plantenga MS (2009) Acute effects of breakfasts containing alpha-lactalbumin, or gelatin with or without added tryptophan, on hunger, 'satiety' hormones and amino acid profiles. *The British journal of nutrition* 101(12): 1859–66.

Onaga T, Zabielski R and Kato S (2002) Multiple regulation of peptide YY secretion in the digestive tract. *Peptides* 23(2): 279–290.

Pannacciulli N, Bunt JC, Koska J, Bogardus C and Krakoff J (2006) Higher fasting plasma concentrations of glucagon-like peptide 1 are associated with higher resting energy expenditure and fat oxidation rates in humans. *The American journal of clinical nutrition* 84(3): 556–60.

Papadimitriou MA, Krzemien AA, Hahn PM and Van Vugt DA (2007) Peptide YY(3-36)-induced inhibition of food intake in female monkeys. *Brain research* 1175: 60–5.

Pasman WJ, Heimerikx J, Rubingh CM, van den Berg R, O'Shea M, Gambelli L, Hendriks HFJ, Einerhand AWC, Scott C, Keizer HG and Mennen LI (2008) The effect of Korean pine nut oil on in vitro CCK release, on appetite sensations and on gut hormones in post-menopausal overweight women. *Lipids in health and disease* 7: 10.

Payne PR and Waterlow JC (1971) Relative energy requirements for maintenance, growth, and physical activity. *The Lancet* 298(7717): 210–211.

Pelkman CL, Chow M, Heinbach R a and Rolls BJ (2001) Short-term effects of a progestational contraceptive drug on food intake, resting energy expenditure, and body weight in young women. *The American journal of clinical nutrition* 73(1): 19–26.

Van Pelt RE, Jones PP, Davy KP, Desouza CA, Tanaka H, Davy BM and Seals DR (1997) Regular exercise and the age-related decline in resting metabolic rate in women. *The Journal of clinical endocrinology and metabolism* 82(10): 3208–12.

Perri F, Pastore MR and Annese V (2006) 13C-octanoic acid breath test for measuring gastric emptying of solids. *European review for medical and pharmacological sciences* 9(5 Suppl 1): 3–8.

Piers LS, Diggavi SN, Rijskamp J, van Raaij JM, Shetty PS and Hautvast JG (1995) Resting metabolic rate and thermic effect of a meal in the follicular and luteal phases of the menstrual cycle in well-nourished Indian women. *The American journal of clinical nutrition* 61(2): 296–302.

PubChem (n.d.) *Octanoic acid*. Available at: http://pubchem.ncbi.nlm.nih.gov/compound/octanoic_acid#section=Top (accessed 17/09/15).

R&D Systems (n.d.) *ELISA Development Guide*. .

Ravussin E, Burnand B, Schutz Y and Jéquier E (1982) Twenty-four-hour energy expenditure and resting metabolic rate in obese, moderately obese, and control subjects. *The American journal of clinical nutrition* 35(3): 566–73.

Riad-Gabriel M, Jinagouda S, Sharma A, Boyadjian R and Saad M (1998) Changes in plasma leptin during the menstrual cycle. *European Journal of Endocrinology* 139(5): 528–531.

Roche (2009a) *Elecsys Oestradiol II Cobas - 3000079122* (V 15.). .

Roche (2009b) *Elecsys Progesterone II Cobas - 12145383122* (V 12.). .

Rosenbaum M and Leibel RL (2014) 20 years of leptin: role of leptin in energy homeostasis in humans. *The Journal of endocrinology* 223(1): T83–96.

Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, Goh VHH, Ridgway EC and Wierman ME (2011) Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids* 76(1-2): 177–82.

Salimetrics (2008) *Collecting unstimulated whole saliva samples by passive drool from human subjects (ages 5+)*. USA.

- Salimetrics (2012a) *Salivary 17 β -Estradiol Enzyme Immunoassay Kit (Item No. 1-3702)*. .
- Salimetrics (2012b) *Salivary Progesterone Enzyme Immunoassay Kit (Item No. 1-1502)*. Suffolk, UK.
- Schommartz B, Ziegler D and Schadewaldt P (1997) Significance of diagnostic parameters in [(13)c]octanoic Acid gastric emptying breath tests. *Isotopes in environmental and health studies*. Taylor & Francis Group 33(1-2): 135–43.
- Schultheiss OC, Dargel A and Rohde W (2003) Implicit motives and gonadal steroid hormones: effects of menstrual cycle phase, oral contraceptive use, and relationship status. *Hormones and Behavior* 43(2): 293–301.
- Schwartz MW, Woods SC, Porte D, Seeley RJ and Baskin DG (2000) Central nervous system control of food intake. *Nature* 404(6778): 661–71.
- Shi H, Seeley RJ and Clegg DJ (2009) Sexual differences in the control of energy homeostasis. *Frontiers in neuroendocrinology*. Elsevier Inc. 30(3): 396–404.
- Shreeve WW, Cerasi E and Luft R (1970) Metabolism of [2-14c] pyruvate in normal, acromegalic and HGH-treated human subjects. *European Journal of Endocrinology* 65(1): 155–169.
- Sino Biological Inc. (n.d.)) *Competitive ELISA: Basic principles*. . Available at: <http://www.elisa-antibody.com/ELISA-Introduction/ELISA-types/competitive-elisa> (accessed 13/10/15).
- Smolin LA and Grosvenor MB (1994) *Nutrition: science and applications*. Saunders College Publishing.
- Soares MJ and Shetty PS (1987) Long-term stability of metabolic rates in young adult males. *Human nutrition. Clinical nutrition* 41(4): 287–90.
- Solomon S, Kurzer M and Calloway D (1982) Menstrual cycle and basal metabolic rate in women. *The American journal of clinical nutrition* 36(4): 611–616.
- Speakman JR and Westerterp KR (2010) Associations between energy demands, physical activity, and body composition in adult humans between 18 and 96 y of age. *The American journal of clinical nutrition* 92(4): 826–34.
- St-Onge M, Mignault D, Allison DB and Rabasa-Lhoret R (2007) Evaluation of a portable device to measure daily energy expenditure in free-living adults. *Am J Clin Nutr* 85(3): 742–749.
- Steiner M, Macdougall M and Brown E (2003) The premenstrual symptoms screening tool (PSST) for clinicians. *Archives of Women's Mental Health* 6(3): 203–209.
- Stewart AA, Marfell-Jones M and Olds T (2011) *International standards for anthropometric assessment*. Lower Hutt, New Zealand: International Society for the Advancement of Kinanthropometry.
- Stoeckel LE, Weller RE, Giddings M and Cox JE (2008) Peptide YY levels are associated with appetite suppression in response to long-chain fatty acids. *Physiology & behavior* 93(1-2): 289–95.
- van Strien T, Frijters JER, Bergers GPA and Defares PB (1986) *The Dutch Eating Behavior*

Questionnaire (DEBQ) for assessment of restrained, emotional, and external eating behavior. *International Journal of Eating Disorders* 5(2): 295–315.

Stubbs RJ, Johnstone AM, O'Reilly LM and Poppitt SD (1998) Methodological issues relating to the measurement of food, energy and nutrient intake in human laboratory-based studies. *The Proceedings of the Nutrition Society* 57(3): 357–72.

Stunkard AJ and Messick S (1985) The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *Journal of Psychosomatic Research* 29(1): 71–83.

Suh S-H, Casazza GA, Horning MA, Miller BF and Brooks GA (2003) Effects of oral contraceptives on glucose flux and substrate oxidation rates during rest and exercise. *Journal of applied physiology* 94(1): 285–94.

Tenovuo JO (1989) *Human Saliva, Volumen 2*. USA: CRC Press, Inc.

Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E and Heiman ML (2001) Circulating ghrelin levels are decreased in human obesity. *Diabetes* 50(4): 707–709.

Uvnäs-Msoberg K, Sjögren C, Westlin L, Anderson PO and Stock S (1989) Plasma Levels of Gastrin, Somatostatin, VIP, Insulin and Oxytocin During the Menstrual Cycle in Women (With and Without Oral Contraceptives). *Acta Obstetrica et Gynecologica Scandinavica* 68(2): 165–169.

de Vries J, Zock P, Mensink R and Katan M (1994) Underestimation of energy intake by 3-d records compared with energy intake to maintain body weight in 269 nonobese adults. *Am J Clin Nutr* 60(6): 855–860.

Van Vugt D a (2010) Brain imaging studies of appetite in the context of obesity and the menstrual cycle. *Human reproduction update* 16(3): 276–92.

Walker RF, Read GF and Riad-fahmy D (1979) Radioimmunoassay of Progesterone in Saliva : Application to the Assessment of Ovarian Function. 2033: 2030–2033.

Wallace M, Hashim YZH-Y, Wingfield M, Culliton M, McAuliffe F, Gibney MJ and Brennan L (2010) Effects of menstrual cycle phase on metabolomic profiles in premenopausal women. *Human reproduction (Oxford, England)* 25(4): 949–56.

Wang Z, Heshka S, Zhang K, Boozer CN and Heymsfield SB (2001) Resting energy expenditure: systematic organization and critique of prediction methods. *Obesity research* 9(5): 331–6.

Webb P (1986) 24-hour energy expenditure and the menstrual cycle. *The American journal of clinical nutrition* 44(5): 614–9.

Weijjs PJM and Vansant GAAM (2010) Validity of predictive equations for resting energy expenditure in Belgian normal weight to morbid obese women. *Clinical nutrition (Edinburgh, Scotland)* 29(3): 347–51.

Weir JB de V. (1949) New methods for calculating metabolic rate with special reference to protein metabolism. *The Journal of physiology* 109(1-2): 1–9.

WHO (2015) Obesity and overweight. World Health Organization.

Willett W (2012) *Nutritional Epidemiology*. .

Worthman CM, Stallings JF and Hofman LF (1990) Sensitive salivary estradiol assay for monitoring ovarian function. *Clinical Chemistry* 36(10): 1769–1773.

Yach D, Stuckler D and Brownell KD (2006) Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nature medicine*. Nature Publishing Group 12(1): 62–6.

Yin X, Li Y, Xu G, An W and Zhang W (2009) Ghrelin fluctuation, what determines its production? *Acta Biochimica et Biophysica Sinica* 41(3): 188–197.

Zimmerman Y, Eijkemans MJC, Coelingh Bennink HJT, Blankenstein MA and Fauser BCJM (2014) The effect of combined oral contraception on testosterone levels in healthy women: a systematic review and meta-analysis. *Human reproduction update* 20(1): 76–105.

Appendices

- UREC Ethical approvals
- Consent forms
- Participant information sheets
- Screening questionnaires
- Life-style questionnaire (includes: PAQ, PSST, TFEQ FI, DEBQ)
- VAS
- 3-day food diary

Dr Helen Lightowler
Senior Lecturer
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Gipsy Lane
Headington

25 February 2013

Dear Dr Lightowler

UREC Registration No: 130697

RMR and hormone levels in saliva and blood during the different phases of the menstrual cycle

Thank you for the recent emails outlining the response to the points raised in my previous letter about the PhD study of your research student **Marta Campolier**, and attaching the revised documents. I am pleased to inform you that, on this basis, I have given Chair's Approval for the study to begin.

The UREC approval period for this study is two years from the date of this letter, so 25 February 2015. If you need the approval to be extended please do contact me nearer the time of expiry.

In order to monitor studies approved by the University Research Ethics Committee, we will ask you to provide a (very brief) report on the conduct and conclusions of the study in a year's time. If the study is completed in less than a year, could you please contact me and I will send you the appropriate guidelines for the report.

Yours sincerely



Hazel Abbott
Chair of the University Research Ethics Committee

cc Sangeetha Thondre, Second supervisor
Marta Campolier, Research Student
Dido Green, Research Ethics Officer
Jill Organ, Graduate Office
Louise Wood, UREC Administrator



UNIVERSITY RESEARCH ETHICS
COMMITTEE, FACULTY OF HEALTH AND
LIFE SCIENCES

Headington Campus Gipsy Lane
Oxford OX3 0BP UK

Tel: 01865 482639
heabbott@brookes.ac.uk

www.brookes.ac.uk

Dr Helen Lightowler
Director of Studies
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Gipsy Lane
Headington

16 October 2014

Dear Dr Lightowler

UREC Registration No: 140854

Appetite hormone response in different phases on the menstrual cycle

Thank you for the email of 14 October 2014 outlining your response to the points raised in my previous letter about the PhD study of your research student Marta Campolier, and attaching the revised documents. I am pleased to inform you that, on this basis, I have given Chair's Approval for the study to begin.

The UREC approval period for this study is two years from the date of this letter, so 16 October 2016. If you need the approval to be extended please do contact me nearer the time of expiry.

Should the recruitment, methodology or data storage change from your original plans, or should any study participants experience adverse physical, psychological, social, legal or economic effects from the research, please inform me with full details as soon as possible.

Yours sincerely

Hazel Abbott
Chair of the University Research Ethics Committee

cc Dr Sangeetha Thondre, Co-investigator
Marta Campolier, Research Student
Dido Green, Research Ethics Officer
Jill Organ, Research Degrees Team
Louise Wood, UREC Administrator

UNIVERSITY RESEARCH ETHICS
COMMITTEE, FACULTY OF HEALTH AND
LIFE SCIENCES

Headington Campus Gipsy Lane
Oxford OX3 0BP UK

Tel: 01865 482639
heabbott@brookes.ac.uk

CONSENT FORM

Resting metabolic rate and hormone levels in saliva and blood during the different phases of the menstrual cycle

Marta Campolier (PhD researcher)

Department of Sport and Health Sciences

Faculty of Health and Life Sciences

Oxford Brookes University

Gipsy Lane Campus

Headington

Oxford OX3 0BP

marta.campolier-2012@brookes.ac.uk

Please initial box

I confirm that I have read and understand the information sheet for the above research project and have had the opportunity to ask questions.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason.

☐

I agree to take part in the above study.

☐

I understand that my data gathered in this study may be stored (after it has been anonymised) in a specialist data centre and may be used for future research.

☐

Name of Participant

Date

Signature

Name of Researcher

Date

Signature

CONSENT FORM

Appetite hormone response in different phases of the menstrual cycle

Mrs Marta Campolier

PhD Researcher
S4.07 Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP
marta.campolier-2012@brookes.ac.uk
Phone: +44 1865 48 3283

Dr Helen Lightowler

Senior Lecturer
S4.06a Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP
hlightowler@brookes.ac.uk
Phone: +44 1865 48 3245

Dr P Sangeetha Thondre

Research Fellow
S4.06a Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP
pthondre@brookes.ac.uk
Phone: +44 1865 48 3988

Please initial box

I confirm that I have read and understand the information sheet for the above research project and have had the opportunity to ask questions.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason.

☐

I agree to take part in the above study.

☐

I understand that my data gathered in this study may be stored (after it has been anonymised) in a specialist data centre and may be used for future research.

Yes

No

☐☐

Name of Participant

Date

Signature

Name of Researcher

Date

Signature

Department of Sport and Health Sciences
Oxford Brookes University, Gypsy Lane, Oxford, OX3 0BP, UK

Marta Campolier

Email: marta.campolier-2012@brookes.ac.uk

Phone: +44 (0) 1865 48 3283

INFORMATION SHEET

Full title of Project: Resting metabolic rate and hormone levels in saliva and blood during different phases of the menstrual cycle

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?

Energy balance (energy input = energy output) is key to maintaining body weight. In contrast, continuous imbalances between what is ingested and “burnt” can induce changes in weight and body composition. Resting metabolic rate (RMR) is the most important component of total energy expenditure. Differences in RMR account for several features e.g. age, gender and body mass index (BMI). While there is a low variation in RMR in men, previous studies have shown wide differences in the variation in RMR in women during the menstrual cycle. These differences may be due to changes in estrogen and progesterone levels during the menstrual cycle. However, limited studies have monitored RMR and hormone levels during the menstrual cycle to fully describe this phenomenon.

Although overweight and obese women have shown to have a higher RMR than lean counterparts, there are no data on RMR throughout phases of the menstrual cycle specifically in this population. Similarly, the use of hormonal contraceptives (HC) and their potential effects on energy metabolism are still ambiguous.

Although blood collection has been usually used in research for measuring hormone levels, saliva samples have become more common to measure and monitor levels of hormones such as cortisol and sex steroids due to being non-invasive, stress-free and easy to collect and store. Although saliva levels have been shown to compare well with hormone levels measured in blood, the ratio between the two specimens seems to be altered between individuals and phases of the menstrual cycle; therefore further research is required.

The aim of this study is to investigate RMR along with ovarian hormone levels during the different phases of the menstrual cycle in healthy women.

Why have I been invited to participate?

We are inviting healthy women volunteers:

- Aged 18-40 years
- With a regular (i.e. cycle length of 25-35 days) and natural or artificial (i.e. induced by hormonal contraceptives) menstrual cycle
- Not pregnant or lactating
- Not having any metabolic/genetic diseases or taking any medications known to interfere with metabolism

Should you agree to participate, and after signing consent, you will then be asked to complete a basic health questionnaire to make sure that you are eligible to participate in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you are a student by choosing to either take part or not take part in the study or to withdraw at any time will have no impact on your marks, assessments or future studies.

What will happen to me if I take part?

After reading this information sheet, we will discuss any queries you may have about the study. Should you then agree to participate, you will be asked to sign a consent form to participate. On an agreed day, you will be asked to come to the Functional Food Centre to complete a screening questionnaire that will give us an indication of your health status, the characteristics of your period and whether you are on any medication that may affect the parameters that we are measuring, thus showing us whether you are eligible for our study.

The general body health profile and menstrual cycle characteristics is for screening purposes only and is not a health check; however this information will be shared with you. The research team may recommend that you see your GP if the information indicates anything unusual about the information collected. They will not be expressing a medical opinion but are indicating that it may be advisable to have a health check with your GP.

Following this screening, if you are eligible to participate we will arrange for you to come to the Functional Food Centre in the mornings for the testing days between 7-10am depending on your convenience.

On the evening before each test day, you will be asked to avoid:

- the consumption of caffeine, alcohol and nicotine
- unusual strenuous exercise that you would not usually undertake as part of your normal 'daily lifestyle'.

You will also have to fast overnight (from 10-12 hours before the testing time).

You will be asked to come to the Functional Food Centre three times a week (e.g. Monday, Wednesday and Friday) for testing for one complete menstrual cycle for a total of ~12 visits (in addition to the initial screening). Optionally, you could participate for an extra menstrual cycle later on.

The following measurements will be taken during each test day after having rested for 20-30 minutes:

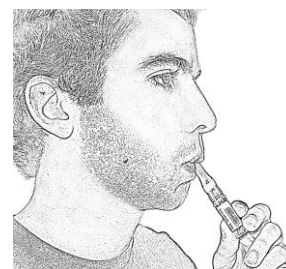
- **Resting metabolic rate (RMR)** – Energy expenditure will be measured while you are lying still under a hood system. The hood is see-through plastic, similar to a hairdryer hood. An image of it can be seen here. The oxygen and carbon dioxide in your breath will be measured and can be used to calculate energy expenditure. You will be asked to lie completely still during the measurement for 30 minutes and the average energy expenditure will be calculated.



During this time you can listen to music or watch DVDs on a laptop.

- **Body composition** - Weight and body composition will be measured by a digital scale (Tanita). Moreover, on the first testing day height will be assessed.

- **Saliva hormones** – 5 ml of unstimulated saliva will be collected by passive drool after having rinsed thoroughly the mouth with water 10 minutes before the sample is collected.
- **Blood hormones** – Forearm blood samples will be taken following the other tests. Each measurement requires only 6ml of blood, therefore we would insert a small needle into a large vein on the under side of the elbow (anti-cubital fosse), it has a tube attached to it which means we can collect blood. After the measurement the needle will be removed and a cotton wool pad will be administered with pressure to prevent any bleeding. All of the procedures will be carried out by a fully trained phlebotomist and be observed by another trained phlebotomist, in addition a first aider will be present, in order to minimise the risk of pain or bleeding, but you should be aware of this risk before deciding to participate.
- Finally on the first testing day in addition to the previously mentioned we will also ask you to fill in a life style questionnaire.



What are the possible disadvantages and risks of taking part?

There are no major risks. The only risks will be sometimes a slight bruising where the blood has been taken. That should disappear within days and both arms will be used alternatively to minimise the bruising. Moreover, it will not prevent you from doing your regular daily activity. Moreover, the participant will be advised to tell any person taking blood in the future that they had fainted as a result of the procedure. In case of repeated severe adverse effects, the participant will be encouraged to withdraw from the study.

The study is quite time consuming as it consists of health and menstrual profile on the preliminary visit as well as the testing days of up to 1½ hours, three times a week during the menstrual cycle; however, as outlined above, you can read, listen to music and watch DVDs during this time.

What are the possible benefits of taking part?

After each testing day you will be provided with a free breakfast once the testing procedures are completed. You will receive £50 per menstrual cycle in Amazon Vouchers and you will also get information from your body composition and menstrual cycle profile. Finally, you will also contribute in furthering our understanding on the topic.

Will what I say in this study be kept confidential?

All information collected about you will be kept strictly confidential (subject to legal limitations). Access to the data will be by the researcher working on this study only. Access to computer files will be by password only and data and the codes and identifying information will be kept in separately in locked filing cabinets.

Data generated by the study must be retained in accordance with the University's policy on Academic Integrity. The data generated in the course of the research must be kept securely in paper or electronic form for a period of ten years after the completion of a research project. Due to the small number of volunteers taking part in this study it may be difficult to maintain anonymity of the volunteers as participants may overlap with their sessions. However all data will be de-identified. Results from the research would be published in a way that looks at the group responses to the questionnaires, so that no individuals would be able to be identified or recognised.

What should I do if I want to take part?

If you would like to take part in this research study you can do so by contacting the researchers at the address, phone number or email address given below.

What will happen to the results of the research study?

The results of this research will be published in a research journal. Your name or identity will not be recognisable from this. If you wish to obtain a copy of the published research you can do so by contacting the researchers at the address, phone number or email address given below following completion of the study. Furthermore, a summary of your personal results will be available to you, if interested.

Who is organising and funding the research?

I am conducting the research as a PhD researcher at Oxford Brookes University under the supervision of Dr Helen Lightowler and Dr Sangeetha Thondre. This research is being completed and funded by the Faculty of Health and Life Science at Oxford Brookes University.

Who has reviewed the study?

This research has been approved by the University Research Ethics Committee at Oxford Brookes University.

Contact for Further Information

Miss Marta Campolier
PhD Researcher
S4.07 Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gypsy Lane, Oxford
OX3 0BP

marta.campolier-2012@brookes.ac.uk

Phone: +44 1865 48 3283

Dr Helen Lightowler
Senior Lecturer
S4.06a Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gypsy Lane, Oxford
OX3 0BP

hlightowler@brookes.ac.uk

Phone: +44 1865 48 3245

Dr P Sangeetha Thondre
Research Fellow
S4.07 Functional Food Centre
School of Life Sciences
Oxford Brookes University
Gypsy Lane, Oxford
OX3 0BP

pthondre@brookes.ac.uk

Phone: +44 1865 48 3988

If you have any concerns about the way in which the study has been conducted, you should contact the Chair of the University Research Ethics Committee on ethics@brookes.ac.uk.

Thank you for taking time to read this information sheet.

Functional Food Centre
Department of Sport and Health Sciences
Oxford Brookes University, Gipsy Lane, Oxford, OX3 0BP, UK

Marta Campolier Email: marta.campolier-2012@brookes.ac.uk

Phone: +44 (0) 1865 483283

INFORMATION SHEET

Full title of Project: Appetite hormone response in different phases of the menstrual cycle

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?

Energy balance (energy input = energy output) is key to maintaining body weight. In contrast, continuous imbalances between what is ingested and “burnt” can induce changes in weight and body composition.

We have observed that some women show modest changes in their energy expenditure, across their menstrual cycle. Moreover, previous research has shown that women might also display different food patterns amongst phases of the menstrual cycle. Nevertheless, little is known regarding what is underpinning these changes. Although the cyclical variations in the sex hormones estrogen and progesterone have been claimed to be responsible for these changes in both sides of the energy balance equation, how these can ultimately induce modifications in the energy consumed and expended still unknown.

Although there are few reports looking at appetite hormones in a fasted state throughout the menstrual cycle, how these may respond to the same breakfast in the different menstrual cycle phases is still uncertain. Previous studies have observed that changes in the emptying rate of foods in the stomach might also be related to changes in appetite hormones.

The aim of this study is to investigate whether appetite hormones and feelings of hunger change at the different phases of the menstrual cycle in healthy women.

Why have I been invited to participate?

We are inviting healthy women volunteers:

- Aged 18-40 years
- With a body mass index (BMI) between 18-30 kg/m²
- With regular (i.e. cycle length of 25-35 days) and natural (i.e. not taking hormonal contraceptives) menstrual cycles
- Not pregnant or lactating
- Not anaemic
- Not having any metabolic/genetic diseases or taking any medications known to interfere with metabolism or appetite
- Not suffering from any eating disorders
- Not following any diet with the attempt to lose weight
- Non-smoking
- Not highly physically active i.e. training more than 5 hours a week, and involved in endurance sports at competitive levels
- Not allergic/intolerant to any of the foods presented in the study
- Habitually consume breakfast and lunch

Should you agree to participate, and after signing the consent form, you will be asked to complete a health questionnaire to make sure that you are eligible to participate in the study.

Do I have to take part?

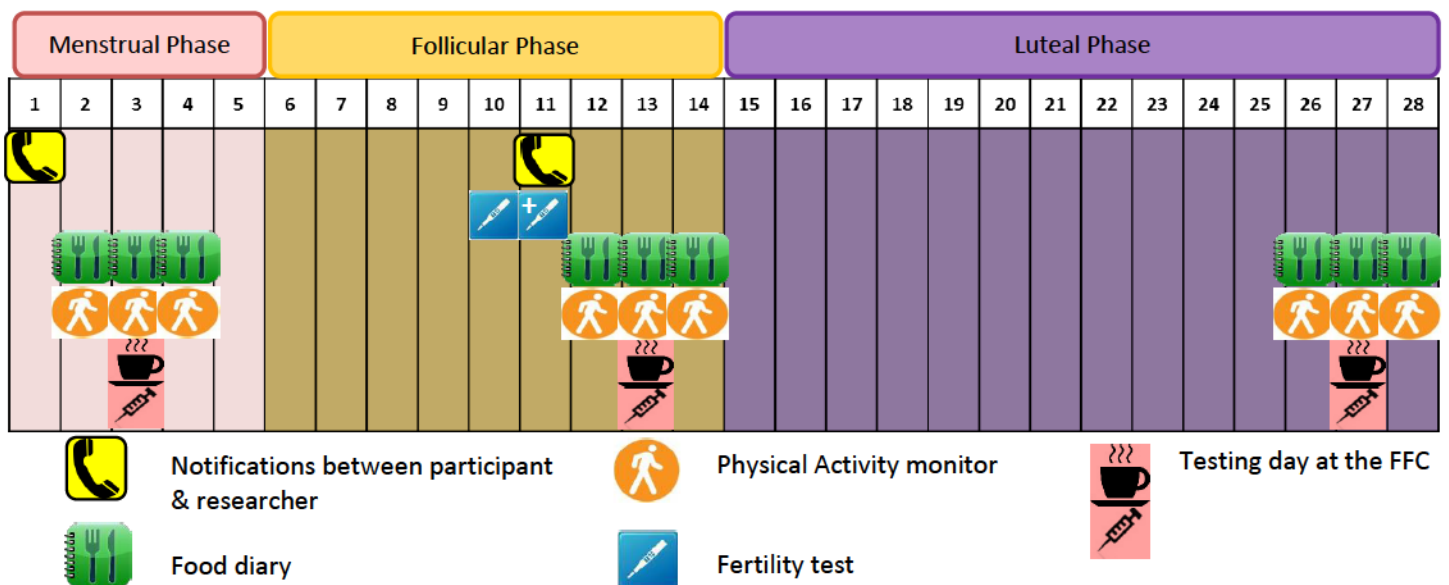
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you are a student by choosing to either take part or not take part in the study or to withdraw at any time will have no impact on your marks, assessments or future studies. If you are a member of staff by choosing to take or not to take part in the study or to withdraw at any time will have no impact on your employment status at the university.

What will happen to me if I take part?

After reading this information sheet and having had at least 24 hours for you to consider your participation, we will discuss any queries you may have about the study. Should you then agree to participate, you will be asked to sign a consent form to participate.

On an agreed day, you will be asked to come to the Functional Food Centre to complete a **screening** and a life style questionnaire with measurements of your body weight that will give us an indication of your health status, the characteristics of your period and whether you are on any medication or condition that may affect the parameters that we are measuring, thus showing us whether you are eligible for our study. The general body health profile and menstrual cycle characteristics is for screening purposes only and is not a health check; however this information will be shared with you. The research team may recommend that you see your GP if the information indicates anything unusual about the information collected. They will not be expressing a medical opinion but are indicating that it may be advisable to have a health check with your GP. If you are eligible to participate we will provide you with a fertility monitor that will help us to schedule the visits to the Functional Food Centre according to the phases of your menstrual cycle. It is estimated that this screening session will last up to 1 hour.

For each cycle phase i.e. menstrual, follicular and luteal, **three consecutive days** according to the fertility monitor readings and the usual length of your menstrual cycle (e.g. days 2 to 4, 12 to 14 and 26 to 28 of your cycle for menstrual, follicular and luteal phases, respectively), will be selected in which you will be asked to **keep record of your food intake** and **wear a body monitoring system** that measures your physical activity. (See example of timeline below)

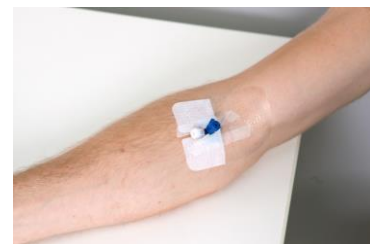


- **Food diary** - you will be asked to weigh and record your food intake in a food diary; during the screening session, you will be provided with food weighing scales to weigh food and beverages and any food waste.
- **Body monitoring system** (SenseWear®) - this consists of an armband that assesses your physical activity throughout the day; you do not need to worry about anything else other than wearing it all times except when you are in constant contact with water e.g. showering, swimming. During the screening session, the SenseWear will be given to you with instructions on how to put it on and use.



The **visit to the Functional Food Centre** will happen on the second of the three selected days in each phase and therefore there will be a total of three visits to the lab for testing for this study. On these visits you will attend the Functional Food Centre after an overnight fast at any time agreed with the researcher between 07:30-09:30 for a total of 5 hours. Each visit will involve the following:

- **Body composition** - Weight and body composition will be measured by a digital scale (Tanita). Moreover, on the first testing day height will be assessed.
- **Breakfast** – You will be provided with a standardized breakfast consisting of: two slices of wholemeal bread toasted with scrambled eggs, a pot of pineapple and tea/coffee.
- **Satiety ratings** – Every 15 minutes for the first hour and every 30 minutes for the following four hours after the breakfast you will be asked to rate on a scale how hungry you are feeling.
- **Gastric emptying** – Breath samples for the measurement of gastric emptying will be taken by blowing into a small glass tube through a straw every 15 minutes for 4 hours after breakfast. Gastric emptying rate will be assessed by the 100 mg of ¹³C sodium acetate added into your breakfast. ¹³C is a naturally abundant stable isotope of carbon that is completely safe and that is regularly used in the food industry as seasoning.
- **Appetite hormones response** – Forearm blood samples will be taken at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes following breakfast. Each measurement requires only 4ml of blood (except at minute 0 which will be 6ml), therefore a total of 46ml of blood in 11 samples will be collected over 4 hours. This may seem a lot but if you were to donate blood, you would give 470ml in 10-15min, therefore the amount drawn for this study is minimal and during a longer period of time. To do this, we will insert a small needle with a cannula into a large vein of your arm and once in place, the needle will be removed so that the only piece left is a small flexible plastic in your arm. a tube will be attached to collect the blood at the abovementioned timings. Moreover, there will be minimal discomfort because after the insertion of the cannula for the first measurement, all further samples do not require the insertion of any needle for their collection. All of the procedures will be carried out by a fully trained phlebotomist. In addition a first aider will be in the area in order to manage any possible adverse event.
- **Sex hormones** – from the first sample drawn before your breakfast, oestrogen and progesterone levels will be also measured to confirm the phase of your menstrual cycle.
- **Buffet lunch** – After the four hours of testing, a buffet lunch will be provided after which you will be free to leave. Foods included in the buffet will range from sandwiches, pizza slices, salads, yogurts, biscuits and fruit, as well as a range of beverages.



Between measurements you will remain in the lab during the entire testing period and you will be allowed to work on your laptop, read or listen to music in a designated area provided for your use.

Finally, on the **evening before** each test day, you will be asked to **avoid**:

- the consumption of caffeine and alcohol
- unusual strenuous exercise that you would not usually do as part of your normal 'daily lifestyle'.

What are the possible disadvantages and risks of taking part?

There are no potential risks to you associated with the proposed research. You will be carefully screened for allergies and intolerances before inclusion in the study. All the foods will be prepared under strict hygiene conditions in a well-managed purpose-built test kitchen. The food ingredients will be carefully monitored for quality and expiry dates. In case of any allergic reaction to the foodstuffs after the testing days, please refer to the NHS or Allergy UK websites given below:

<http://www.nhs.uk/conditions/food-allergy/pages/intro1.aspx>

<http://www.allergyuk.org/corporate.aspx> or dial Allergy UK helpline at 01322 619898.

The cannulation procedure can feel a bit uncomfortable during the insertion of the cannula however, that should not hurt you during the 4 hours that you will be wearing it, nor during the collection of each blood sample. In case of an unsuccessful cannulation, the researcher will consider seeking assistance/advice from a more experienced member of staff. No more than a total of four attempts will be made and only with your full consent before each attempt. In case of having fainted during the procedure, you will be advised to tell the person taking blood in the future and if repeated adverse effects occur you will be encouraged to withdraw from the study. Hygiene and good practice guidelines will be followed at all times to reduce any little chance of getting your vein infected or inflamed. Under the unexpected occurrence of any of these, the cannula will be removed immediately and you will be advised to see your GP.

The study is quite time consuming as it consists of health and menstrual profile on the preliminary visit as well as the three testing days of up to 5 hours; however, as outlined above, you can read, work on your laptop, listen to music and watch DVDs during this time.

What are the possible benefits of taking part?

For every testing day you will have a free breakfast and lunch. Moreover, you will be able to get information about your menstrual cycle and body composition and you will receive £50 Amazon Voucher after the completion of the study. Finally, you will also contribute in furthering our understanding on the topic.

Will what I say in this study be kept confidential?

All information collected about you will be kept strictly confidential (subject to legal limitations). Access to the data will be by the researcher working on this study only. Access to computer files will be by password only and data and the codes and identifying information will be kept in separately in locked filing cabinets.

Data generated by the study must be retained in accordance with the University's policy on Academic Integrity. The data generated in the course of the research must be kept securely in paper or electronic form for a period of ten years after the completion of a research project. Due to the small number of volunteers taking part in this study it may be difficult to maintain anonymity of the volunteers as participants may overlap with their sessions. However all data will be de-identified, i.e. a reversible process in which your name will be removed and replaced by a code that will be used by those handling your data. If necessary, it will be possible to link the code to the original identifiers and identify your data but these will be kept separately and securely with only access to the researcher.

Results from the research would be published in a way that looks at the group responses to the questionnaires, so that no individuals would be able to be identified or recognised. Moreover, plasma samples stored (after having been anonymised) may be used for future research.

What should I do if I want to take part?

If you would like to take part in this research study you can do so by contacting the researchers at the address, phone number or email address given below.

What will happen to the results of the research study?

The results of this research will be published in a research journal. Your name or identity will not be recognisable from this. If you wish to obtain a copy of the published research you can do so by contacting the researchers at the address, phone number or email address given below following completion of the study. Furthermore, a summary of your personal results will be available to you, if interested.

Who is organising and funding the research?

I am conducting the research as a PhD researcher at Oxford Brookes University under the supervision of Dr Helen Lightowler and Dr Sangeetha Thondre. This research is being completed and funded by the Faculty of Health and Life Science at Oxford Brookes University.

Who has reviewed the study?

This research has been approved by the University Research Ethics Committee at Oxford Brookes University. If you have any concerns about the conduct of this research project you can contact the Chair of the University Research Ethics Committee at Oxford Brookes University, at the e-mail address: ethics@brookes.ac.uk

Contact for Further Information

Miss Marta Campolier
PhD Researcher
S4.07 Functional Food
Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP

[marta.campolier-
2012@brookes.ac.uk](mailto:marta.campolier-2012@brookes.ac.uk)

Phone: +44 1865 48 3283

Dr Helen Lightowler
Senior Lecturer
S4.06a Functional Food
Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP

hlightowler@brookes.ac.uk

Phone: +44 1865 48 3245

Dr P Sangeetha Thondre
Research Fellow
S4.06a Functional Food
Centre
School of Life Sciences
Oxford Brookes University
Gipsy Lane, Oxford
OX3 0BP

pthondre@brookes.ac.uk

Phone: +44 1865 48 3988

If you have any concerns about the way in which the study has been conducted, you should contact the Chair of the University Research Ethics Committee on ethics@brookes.ac.uk.

Thank you for taking time to read this information sheet.

Date: ____ / ____ / ____

ID: ____

Screening Questionnaire

Please answer all questions with a circle or as appropriate. If you have a problems with this questionnaire please ask.

General Health

1. When were you born? ____ / ____ / ____ (DD/MM/YYYY)
2. Are you allergic to any foods? Yes No
If yes which one(s)? _____
3. Do you have a genetic or metabolic disease? Yes No
If yes which one(s)? _____
4. Do you suffer from anaemia? Yes No
5. Are you taking any medication including herbal? Yes No
If yes, which one(s)? _____
6. Are you a smoker? Yes No
If yes, how many a day? _____ cigarettes/day

Menstrual Cycle

1. What is the average length of your menstrual cycle? (i.e. from beginning of one menstruation to the beginning of the next) _____ days
2. What is the average length of your menstruation? (i.e. bleeding days) _____ days
3. Number of days since the start of your last menstruation? _____ days
4. Have you experienced regular menstrual cycles for the previous 3 cycles? Yes No
5. Do you use any hormonal contraceptive? Yes No
If yes, which kind of? (e.g. type, brand) _____
And for how long have you been using them? _____
6. Have you given birth within the last 2 years? Yes No
If yes, are you still lactating / breastfeeding? Yes No
If not, when did you stop? _____

**When you have finished please hand back to the researcher.
THANK YOU FOR YOUR TIME**

Official use only

Breakfast: Cereals | Bread | Self-provided |

Next expected period: _____

Testing time slot: ____ : ____

Date: ____ / ____ / ____

ID: ____

Screening Questionnaire

Please answer all questions with a circle or as appropriate. If you have a problems with this questionnaire please ask.

General Health

1. When were you born? ____ / ____ / ____ (DD/MM/YYYY)
2. Are you allergic to any foods? Yes No
If yes which one(s)? _____
3. Are you following a diet with the aim to put on or lose weight? Yes No
4. Do you habitually consume breakfast and lunch? Yes No
5. Do you have a genetic or metabolic disease? Yes No
If yes which one(s)? _____
6. Do you suffer from anaemia? Yes No
7. Are you taking any medication including herbal? Yes No
If yes, which one(s)? _____
8. Are you a smoker? Yes No

Menstrual Cycle

1. What is the average length of your menstrual cycle? (*i.e. from beginning of one menstruation to the beginning of the next*) _____ days
2. What is the average length of your menstruation? (*i.e. bleeding days*) _____ days
3. What date did your last menstruation start? ____ / ____ / ____
4. Have you experienced regular menstrual cycles for the previous 3 cycles? Yes No
5. Do you use any hormonal contraceptive? Yes No
6. Have you given birth within the last 2 years? Yes No
If yes, are you still lactating / breastfeeding? Yes No
If not, when did you stop? _____

**When you have finished please hand back to the researcher.
THANK YOU FOR YOUR TIME**

Body Composition

Height
Weight
Tanita:
• BMI
• % Fat Mass
• Fat Mass (kg)
• Fat Free Mass
• Total Body Water
Waist (cm)
Hip (cm)
WHR

Equipment Provided

Food Scales
PA Monitor
Fertility Monitor

Breakfast Details

Coffee	<input type="checkbox"/>	Milk	<input type="checkbox"/>
Tea	<input type="checkbox"/>	Sugar	<input type="checkbox"/>
Water	<input type="checkbox"/>		
Time			

Menstrual Cycle Dates	Date	Cycle Day
Start Cycle		
End Menses		
High Phase		
Peak Days		
Testing Days	MPh	
	FPh	
	LPh	
End Cycle		

Cannula Site

Screening
MPh
FPh
LPh

0:00

(min 0)

How hungry do you feel?

Not at all hungry Extremely hungry

How full do you feel?

Not at all full Extremely full

How strong is your desire to eat?

Not at all strong Extremely strong

How much food do you think you can eat?

A large amount Nothing at all

0:15

(min 15)

How hungry do you feel?

Not at all hungry Extremely hungry

How full do you feel?

Not at all full Extremely full

How strong is your desire to eat?

Not at all strong Extremely strong

How much food do you think you can eat?

A large amount Nothing at all

0:30

(min 30)

How hungry do you feel?

Not at all hungry Extremely hungry

How full do you feel?

Not at all full Extremely full

How strong is your desire to eat?

Not at all strong Extremely strong

How much food do you think you can eat?

A large amount Nothing at all

0:45

(min 45)

How hungry do you feel?

Not at all hungry | Extremely hungry

How full do you feel?

Not at all full | Extremely full

How strong is your desire to eat?

Not at all strong | Extremely strong

How much food do you think you can eat?

A large amount | Nothing at all

1:00

(min 60)

How hungry do you feel?

Not at all hungry | Extremely hungry

How full do you feel?

Not at all full | Extremely full

How strong is your desire to eat?

Not at all strong | Extremely strong

How much food do you think you can eat?

A large amount | Nothing at all

1:30

(min 90)

How hungry do you feel?

Not at all hungry | Extremely hungry

How full do you feel?

Not at all full | Extremely full

How strong is your desire to eat?

Not at all strong | Extremely strong

How much food do you think you can eat?

A large amount | Nothing at all

(min 120)

A large amount Nothing at all

(min 150)

A large amount

Nothing at all

(min 180)

A large amount Nothing at all

3:30

(min 210)

How hungry do you feel?

Not at all hungry | Extremely hungry

How full do you feel?

Not at all full | Extremely full

How strong is your desire to eat?

Not at all strong | Extremely strong

How much food do you think you can eat?

A large amount | Nothing at all

4:00

(min 240)

How hungry do you feel?

Not at all hungry | Extremely hungry

How full do you feel?

Not at all full | Extremely full

How strong is your desire to eat?

Not at all strong | Extremely strong

How much food do you think you can eat?

A large amount | Nothing at all

Food Diary

Participant ID: _____

Days Recorded:

1. ____ / ____ / ____
2. ____ / ____ / ____
3. ____ / ____ / ____

Cycle day: ____

Cycle day: ____

Cycle day: ____

Food Diary

We would like you to keep a diary of everything you eat and drink over 3 days. Please read carefully the instructions below before starting to fill in the diary.

It is important to **record everything you eat and drink**, no matter how small the amount.

- Each day is marked in 6 sections, beginning with the first thing in the morning and ending with bedtime. For each part of the day, write down all food and drink consumed. If nothing is consumed during a part of the day draw a line through that section.
- Give **as much information as possible** about the foods and drinks you eat. It is very useful if you include:
 - Brand name e.g. Branston pickle,
 - How the food was cooked, e.g. baked, grilled, raw, etc.
 - Extra ingredients, e.g. teaspoon of grated parmesan on pasta or dressings/sauces added to salads
- Estimating the amount eaten:
 - **Use the scale** provided to record food weights when possible
 - Look at the details on the packaging of some foods
 - Household measures may help;
 - A general recording of whether portions are small, medium or large is also helpful.
- Remembering later is often more difficult than we initially predict, so it is often easier to **try to record as you go through the day**, where possible.
- Please start **each day on a new sheet**
- See the example on the following page

DATE	0	3	1	2	1	4	Day of the week:	Wednesday
BEFORE BREAKFAST								
Time	Food/Drink		Description & Preparation				Amount	
— : —								
BREAKFAST								
Time	Food/Drink		Description & Preparation				Amount	
<u>07 : 30</u>	Cornflakes		Kellogg's, Special K				1 Bowl	
	Milk		Whole (Cravendale)				200 g	
	Sugar		Siucra				15 g	
MID-MORNING (between breakfast and lunch)								
Time	Food/Drink		Description & Preparation				Amount	
<u>10 : 45</u>	Toast		White, Warburton's				1 slice	
			Crusty, thick					
	Butter		Anchor				50 g	
LUNCH								
Time	Food/Drink		Description & Preparation				Amount	
<u>13 : 30</u>	Bread roll		French style baguette				1 plate	
	Butter		Anchor				50 g	
	Chicken		Breast pieces grilled				250 g	
	Lettuce		Iceberg				30 g	
	Tomato		Cherry				3 small	

Food Diary

DATE								Day of the week:		
BEFORE BREAKFAST										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
BREAKFAST										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
MID-MORNING (between breakfast and lunch)										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
LUNCH										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										

Time	Food/Drink	Description & Preparation	Amount
TEA (between lunch and evening meal)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
EVENING MEAL			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
AFTER EVENING MEAL (between evening meal and bedtime)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			

Food Diary

DATE									Day of the week:		
BEFORE BREAKFAST											
Time	Food/Drink		Description & Preparation						Amount		
___ : ___											
BREAKFAST											
Time	Food/Drink		Description & Preparation						Amount		
___ : ___											
MID-MORNING (between breakfast and lunch)											
Time	Food/Drink		Description & Preparation						Amount		
___ : ___											
LUNCH											
Time	Food/Drink		Description & Preparation						Amount		
___ : ___											

Time	Food/Drink	Description & Preparation	Amount
___ : ___			
TEA (between lunch and evening meal)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
EVENING MEAL			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
AFTER EVENING MEAL (between evening meal and bedtime)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			

Food Diary

DATE									Day of the week:	
BEFORE BREAKFAST										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
BREAKFAST										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
MID-MORNING (between breakfast and lunch)										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										
LUNCH										
Time	Food/Drink		Description & Preparation				Amount			
___ : ___										

Time	Food/Drink	Description & Preparation	Amount
TEA (between lunch and evening meal)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
EVENING MEAL			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			
AFTER EVENING MEAL (between evening meal and bedtime)			
Time	Food/Drink	Description & Preparation	Amount
___ : ___			